# The lignan macromolecule from flaxseed Structure and bioconversion of lignans 

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## Proefschrift

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#### Abstract

Lignans are diphenolic compounds, which are of interest because of their positive health effects. The aims of the research described in this thesis are to identify the precise composition and structure of the lignan macromolecule from flaxseeds, to convert plant lignans into the bioactive mammalian lignans by fermentation, and to investigate how the bioconversion of lignans influences their estrogenicity. In order to be able to reach these goals, analytical and preparative protocols were developed. The lignan macromolecule from flaxseed was found to consist of mainly secoisolariciresinol diglucoside (SDG) ester-linked via 3-hydroxy-3-methylglutaric acid (HMGA). Furthermore, the hydroxycinnamic acid glucosides, coumaric acid glucoside (CouAG) and ferulic acid glucoside (FeAG) were identified as constituents as well. The flavonoid herbactin diglucoside (HDG) was identified as a novel constituent of the lignan macromolecule. HDG is, just as SDG, esterified within the lignan macromolecule via HMGA. CouAG was found to link to the C-6 of a glucosyl moiety of SDG, and FeA to the $\mathrm{C}-2$. The results suggested that CouAG and FeAG are mainly present at the terminal positions and, therefore, it was hypothesized that they determine the chain length of the lignan macromolecule. By modeling the relationship between the composition and the size it was shown that the average lignan macromolecule consists of three SDG units with two hydroxycinnamic acid glucosides at the terminal positions. Lignans were converted to the mammalian lignans by fermentation. Secoisolariciresinol (SECO) and its dehydrated derivative, anhydrosecoisolariciresinol (AHS), were demethylated in a similar way by Peptostreptococcus productus, Eubacterium limosum and Clostridium methoxybenzovorans. The dehydroxylation reaction was found to be more specific. Demethylated SECO could be dehydroxylated completely by Eggerthella lenta. Demethylated AHS was dehydroxylated to a lower extent than demethylated SECO. The products obtained by fermentation were purified by preparative RP-HPLC loading the samples by at-column-dilution. Furthermore, lignans from sesame seeds were investigated. Only by combining APCI-MS in the positive and the negative ionization mode, all sesame-derived lignans in complex (fermentation) mixtures could be identified. In conclusion, this research resulted in a more detailed description of the lignan macromolecule from flaxseeds. Two series of structurally different lignans were obtained by bacterial conversion.


Keywords: lignan, lignan macromolecule, secoisolariciresinol, anhydrosecoisolariciresinol, enterodiol, enterolactone, flaxseed, sesame seed, bioconversion, fermentation, demethylation, dehydroxylation, estrogenicity, RP-HPLC-MS, preparative RP-HPLC


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## Lignans

"Functional foods", "nutraceuticals" or "pharmaconutrients" are all terms, which indicate nutrients or nutrient-enriched foods that can prevent or treat diseases (Hardy, 2000). These foods and food components represent the fastest growing segment in food industry (Andlauer and Furst, 2002). In this thesis, one of the many nutraceutical compounds, the lignans, are investigated.
The consumption of lignans in The Netherlands varies between $43 \mu \mathrm{~g}$ and 8 mg per day, with a median of almost 1 mg per day (Milder et al., 2005b). The major sources of lignans in the human diet are oilseeds, nuts and grains, together with vegetables and tea (Milder et al., 2005b). In Table 1 an overview is given of several foods with their lignan contents. This research focuses on the two oilseeds with the highest concentrations of lignans: flaxseeds and sesame seeds.

Table 1. Lignan content of a selection of food sources.

| Source | Total lignan content ( $\mu \mathrm{g} / 100 \mathrm{~g}$ fresh weight) | Major lignan | Reference |
| :---: | :---: | :---: | :---: |
| Flaxseed | 301129-370987 | secoisolariciresinol | (Mazur et al., 1996; |
|  |  |  | Milder et al., 2005a) |
| Sesame seeds | 138365-581000 | sesamin | (Moazzami and Kamal-Eldin, 2006; Smeds et al., 2007) |
| Sunflower seed | 891 | lariciresinol | (Milder et al., 2005a) |
| Soy flower | 130 | secoisolariciresinol | (Mazur et al., 1998) |
| Pea (jar) | 34 | pinoresinol | (Milder et al., 2005a) |
| Brown bean (jar) | 26 | lariciresinol | (Milder et al., 2005a) |
| Rye | 10377 | syringaresinol | (Smeds et al., 2007) |
| Wheat | 7548 | 7-hydroxymatairesinol | (Smeds et al., 2007) |
| Barley | 1071 | 7-hydroxymatairesinol | (Smeds et al., 2007) |
| Corn | 1049 | 7-hydroxymatairesinol | (Smeds et al., 2007) |
| Broccoli | 1325 | lariciresinol | (Milder et al., 2005a) |
| Green bean | 273 | lariciresinol | (Milder et al., 2005a) |
| Bell peper (red) | 113 | lariciresinol | (Milder et al., 2005a) |
| Tomato | 58 | lariciresinol | (Milder et al., 2005a) |
| Potato (boiled) | 20 | lariciresinol | (Milder et al., 2005a) |
| Lettuce (Iceberg) | 11 | secoisolariciresinol | (Milder et al., 2005a) |
| Strawberry | 334-1578 | pinoresinol; secoisolariciresinol | (Meagher and Beecher, 2000; Milder et al., 2005a) |
| Orange | 78 | lariciresinol | (Milder et al., 2005a) |
| Apple (Elstar) | 1 | lariciresinol | (Milder et al., 2005a) |
| Tea (English blend) | 71 | pinoresinol | (Milder et al., 2005a) |
| Coffee (brewed) | 19 | secoisolariciresinol; lariciresinol | (Milder et al., 2005a) |

## Nomenclature

Lignans and neolignans are diphenolic compounds, which consist of two propyl benzene (C6-C3) structures derived from the phenylpropanoid pathway (Moss, 2000). They comprise a whole class of compounds with a similar basic skeleton, but with large variations in substitution patterns.

Lignans are defined as compounds in which the two C6-C3 units are linked to each other via the C8 and the C8` (see Fig. 1 for numbering)(Moss, 2000). Neolignans on the other hand, are composed of two C6-C3 units, which are linked via other carbon atoms than the C8. Within the neolignans, three subgroups are distinguished: the oxyneolignans, the sesquineolignans and the dineolignans. The oxyneolignans have their C6-C3 units linked via an ether linkage. The sesquineolignans are composed of three C6-C3 units and the dineolignans of four C6-C3 units (Moss, 2000).

Lignans have been identified in many plants (Umezawa, 2003), while neolignans are thought to be less abundant and mainly present in coniferous trees (Suzuki and Umezawa, 2007). In edible plant sources, almost exclusively lignans have been found (Milder et al., 2005a; Smeds et al., 2007). Because of the low abundance of neolignans in food sources, this group of compounds will not be not discussed further.


Figure 1. Basic chemical structure of lignans and neolignans.

## Biosynthesis of lignans

## Phenylpropanoid pathway

The basic units of lignans, the C6-C3 units, are formed by the phenylpropanoid pathway starting from phenylalanine. Also lignin and flavonoids are derived from this pathway. The phenylpropanoid pathway is schematically depicted in Fig. 2 (Boerjan et al., 2003; Costa et al., 2003; Davin and Lewis, 1992).

The first step of the phenylpropanoid pathway is the deamination of phenylalanine yielding cinnamic acid. Phenylalanine-ammonia lyase, the enzyme responsible for the deamination, is speculated to play a regulatory role in biosynthesis. Next, cinnamic acid is oxidized to $p$ coumaric acid. Further oxidation of $p$-coumaric acid results in the formation of caffeic acid, which can be further metabolized into consecutively ferulic acid, 5-hydroxyferulic acid and sinapic acid. These hydroxycinnamic acids are converted into their coenzyme-A activated forms, which are further reduced to their aldehydic forms and finally into alcohols (Boerjan et al., 2003; Costa et al., 2003; Davin and Lewis, 1992).
The enzymes catalyzing the various reaction steps regulate the distribution of monomeric units over the various classes of compounds derived from the phenylpropanoid pathway including the hydroxycinnamic acids, flavonoids, lignin and lignans (Davin and Lewis, 1992).


Figure 2. Phenylpropanoid pathway. PAL $=$ phenylalanine ammonium lyase, $\mathrm{C} 4 \mathrm{H}=$ cinnamate 4hydroxylase, 4CL $=4$-coumarate:CoA ligase, CCR = cinnamoyl-CoA reductase, CAD = cinnamyl alcohol dehydrogenase, $\mathrm{SAD}=$ sinapyl alcohol dehydrogenase, COMT $=$ caffeic acid $O$ methyltransferase, CoCoAOMT = caffeoyl-CoA O-methyltransferase, F5H = ferulate 5-hydroxylase, dotted arrow $=$ direct conversion not demonstrated, ? = conversion has been demonstrated but enzyme not indentified. Figure derived from Boerjan et al. (2003).

## Stereospecific coupling by dirigent proteins

First indications for the formation of lignans via stereoselective coupling of two coniferyl alcohols came from experiments with Forsythia species. When two C6-C3 moieties were oxidized in the presence of the insoluble fraction of the Forsythia stem, only (+)-pinoresinol (PINO) was formed (Davin et al., 1997; Umezawa et al., 1990).
From the insoluble fraction of the Forsythia stem, a protein was purified, which catalyzed the dimerisation of trans-coniferyl alcohol, but only in the presence of an oxidase (e.g. laccase). This protein was called dirigent protein since it aligns the dimerisation, and is not involved in the reaction itself (Davin et al., 1997). The dirigent proteins have been found to be widely distributed over the plant kingdom (Gang et al., 1999).

The working mechanism of the dirigent proteins is shown in Fig. 3. Oxidant activity (non specific oxidase or chemical oxidant) results in the formation of radicals, which are aligned by the dirigent protein followed by the stereoselective radical coupling reaction (Davin et al., 1997; Halls et al., 2004). Capturing of the coniferyl alcohol radicals by the dirigent protein makes that the two C6-C3 moieties are aligned in such a way that the formation of only one stereoisomer is possible. Consequently, there are different dirigent proteins for the formation of (+) and (-)-PINO (Davin and Lewis, 2000). The dirigent proteins are also substrate specific. Of the C6-C3 moieties formed in the phenylpropanoid pathway, only coniferyl alcohol is dimerized in a stereospecific way (Davin et al., 1997).
After dimerization, furan rings are being formed by spontaneous intramolecular cyclization driven by reactive quinone methide moieties. This results in the formation of a single stereoisomer of PINO.


Figure 3. Dirigent protein mediated stereoselective radical coupling of coniferyl radicals (adapted from (Davin and Lewis, 2005)).

After the formation of PINO, the lignan biosynthesis branches into two major pathways (Fig. 4). In the first pathway, the furan structures are reduced, which leads to the formation of dibenzylbutane lignans. In the second branch, the furan structures remain intact and the methylenedioxybridged furanofuran lignans are formed.

## Biosynthesis of dibenzylbutane lignans

During the biosynthesis of dibenzylbutane lignans, PINO is stereoselectively converted into consecutively lariciresinol (LARI), secoisolarisiresinol (SECO), and matairesinol (MAT) (Fig. 4). Two enzymes are involved in these reaction steps. Pinoresinol/lariciresinol reductase (PLR) mediates the NADPH dependent reductive cleavage of the furan rings yielding SECO (Dinkova-Kostova et al., 1996). Secoisolariciresinol dehydrogenase (SDH) catalyzes the $\mathrm{NAD}^{+}$dependent and enantiospecific conversion of SECO to MAT (Xia et al., 2001). Since both enzymes work in a stereospecific fashion, the stereochemistry of lignans is determined by the combined action of these two enzymes and the dirigent protein.


Figure 4. Biosynthetic pathways of dibenzylbutane lignans and methylenedioxybridged furanofuran lignans starting from PINO. The enzymes involved determine the stereochemistry of the lignans. PLR $=$ pinoresinol-lariciresinol reductase, $\mathrm{SDH}=$ secoisolariciresinol dehydrogenase, PSS $=$ piperitolsesamin synthase. Dotted arrow = putative biosynthetic pathway.

PLR has been reported to first completely convert PINO into LARI before LARI is converted to SECO (Hemmati et al., 2007; von Heimendahl et al., 2005). In plants, several PLRs have been identified, which differ in enantioselectivity (Dinkova-Kostova et al., 1996; von Heimendahl et al., 2005). The precise topology of the binding pocket of PLRs seems to dictate the binding of either the (+) or (-)-antipode (Min et al., 2003). So, depending on which PLRs are expressed, (+)- or (-)-PINO can be converted into (-)- or (+)SECO (Dinkova-Kostova et al., 1996; Hemmati et al., 2007; von Heimendahl et al., 2005). In flaxseed, for example, two different dirigent proteins and several PLR genes have been identified (von Heimendahl et al., 2005), which explains that besides the predominant (+)-
antipode of SECO also the (-)-isomer can be identified (Ford et al., 2001). In Forsythia, on the other hand, (-)-SECO is the only isomer identified. Recently, a new enzyme, pinoresinol reductase, has been identified. This enzyme only performs a single reduction, and consequently converts PINO to LARI. Furthermore, this enzyme has a less strict enantioselectivity than PLR (Nakatsubo et al., 2008).
Secoisolariciresinol dehydrogenase (SDH) catalyzes the conversion of SECO to matairesinol (MAT) with a lactol as intermediate product (Xia et al., 2001). Also in case of SDH, the topology of the binding pocket determines the stereospecificity (Moinuddin et al., 2006; Youn et al., 2005).
MAT can, dependent on the plant species, be further metabolized (Moinuddin et al., 2006; von Heimendahl et al., 2005; Xia et al., 2000). For instance, podophyllotoxin, a well-known therapeutic agent from e.g. Linum flavum, can be formed (Xia et al., 2000). Besides, lignans can be glucosylated. Secoisolariciresinol glucosyltransferase has been suggested to be the enzyme catalyzing the glucosylation of SECO (Ford et al., 2001).

## Biosynthesis of methylenedioxybridged furanofuran lignans

PINO is also the precursor of the methylenedioxybridged furanofuran lignans (Fig. 4). Most research on this biosynthetic pathway is done in sesame. Piperitol/sesamin synthase (PSS), a member of the P450 enzyme family, catalyzes the conversion of (+)-PINO into consecutively (+)-piperitol and (+)-sesamin (Jiao et al., 1998; Ono et al., 2006). Thus, PSS is responsible for the formation of the methylenedioxybridges.
Sesamin is further converted into sesamolin with epi-sesaminone as possible intermediate (Jiao et al., 1998). The enzymes responsible for this reaction have not been identified yet. The biosynthetic route to another important furanofuran lignan, sesaminol, is unknown. Sesaminol can be glucosylated by glucosyltransferases (Noguchi et al., 2008).

From the above it is clear that the repertoire of enzymes involved in lignan biosynthesis results in many different lignan structures. Lignan diversity is increased even more by the fact that the majority of these enzymes work in a stereospecific way, and by the action of glycosyltransferases.

## Lignans and nutrition

Several health beneficial effects are attributed to flaxseeds and sesame seeds. The beneficial effects of these seeds are often related to their lignan content, even though they also have a beneficial fatty acid composition and a high fiber content (see Table 2 for the nutrient composition) (Hall et al., 2006; Namiki, 2007). Health benefits as protection against certain types of cancer and protection against osteoporosis, are often ascribed to lignans (Adlercreutz, 2002; Kiberstis et al., 2000; Webb and McCullough, 2005). The main mechanisms via which lignans exert their health effects are by influencing the estrogen metabolism and by their antioxidant activity. Because of their influence on estrogen metabolism, lignans are functionally classified as phytoestrogens.

Chapter 1
Table 2. Average nutrient composition of flaxseeds and sesame seeds.

|  | Flaxseed $(\mathrm{g} / 100 \mathrm{~g})^{1}$ | Sesame seed $(\mathrm{g} / 100 \mathrm{~g})^{2}$ |
| :--- | :---: | :---: |
| Carbohydrates | 34 | 24 |
| Protein | 21 | 20 |
| Triglycerides, of which | 41 | 52 |
| $\quad$ saturated | 4 | 8 |
| mono-unsaturated | 7 | 22 |
| poly-unsaturated | 30 | 22 |
| Vitamins and minerals | 2.2 | 3.5 |
| Lignans | 1.8 | 0.4 |
| ${ }^{1}$ derived from (Vaisey-Genser and Morris, 1997) |  |  |
| ${ }^{2}$ derived from (Namiki, 1995; Namiki, 2007) |  |  |

## Health effects of flaxseeds

Consumption of flaxseeds is related to protective effects against some hormone-related types of cancer like breast cancer and prostate cancer. The research done on breast cancer (including mammary gland cancer) in rats and mice shows a reduction in tumor development, tumor size, tumor growth and metastasis upon exposure to flaxseed (Chen et al., 2007; Chen et al., 2002; Chen et al., 2003; Chen et al., 2004; Jungestrom et al., 2007; Thompson et al., 1996; Wang et al., 2005). This protective effect is confirmed a by human intervention study (Thompson et al., 2005).
The effects of flaxseeds on prostate cancer are less pronounced. Flaxseed inhibited the growth and development of prostatic carcinoma in mice (Lin, 2002). However, in human studies, no clear protective effects flaxseed were observed (Demark-Wahnefried et al., 2004).

Flaxseed has been shown to reduce the aberrant crypts (early marker of colon cancer) and to prevent colon tumor development in rat studies (Bommareddy et al., 2006; Jenab and Thompson, 1996; Serraino and Thompson, 1992) due to, in part, its lignan content (Jenab and Thompson, 1996). However, studies with mice or humans did not show any and sometimes even a slightly negative effect of flaxseed on the development of colon cancers (Hallund et al., 2006; Kranen et al., 2003; Oikarinen et al., 2005).
During menopause, the level of estradiol drops. This drop in estrogen level is related to a higher incidence of osteoporosis in post-menopausal women (Kiberstis et al., 2000). Phytoestrogens are shown to reduce this increase in osteoporosis (Picherit et al., 2000), although the effect of lignans is not completely clear yet (Power et al., 2006; Ward et al., 2001). Also the effects of flaxseed on other menopausal discomforts like hot flushes need more investigation (Lewis et al., 2006).
Flaxseed lignans have also been related to protection against cardiovascular diseases. A reduction of plasma cholesterol and prevention against atherosclerotic lesions have been observed (Lucas et al., 2004; Prasad, 1997; Zhang et al., 2008). A high consumption of flaxseeds is also suggested to result in a protective effect against diabetes mellitus (Pan et al., 2007; Zhang et al., 2008).

A



(+)-matairesinol

isolariciresinol
(-)-lariciresinol

B




coumaric acid caffeic acid
ferulic acid


sinapic acid





Figure 5. (A) Chemical structures of lignans from flaxseed (Bakke and Klosterman, 1956; Liggins et al., 2000; Meagher et al., 1999; Sicilia et al., 2003). The stereochemistry of isolariciresinol has not been identified. (B) Chemical structures of other phenolic compounds from flaxseed (Dabrowski and Sosulski, 1984; Hall et al., 2006; Klosterman et al., 1955; Qiu et al., 1999).

## Lignans from flaxseeds

The health effects of flaxseeds are often related to the estrogenic properties of its lignans. Several lignans, mainly dibenzylbutane type lignans, are described in flaxseed as depicted in Fig. 5A. Besides lignans, other compounds derived from the phenylpropanoid pathway, such as hydroxycinnamic acids and flavonoids, have been identified in flaxseeds (Fig. 5B). The lignans, hydroxycinnamic acids and flavonoids are mainly present as glycosides.

Secoisolariciresinol diglucoside (SDG), the main lignan in flaxseed, is hardly present in an unbound from (Hano et al., 2006), but is linked in a macromolecular structure, called the lignan macromolecule or lignan complex (Kamal-Eldin et al., 2001), as represented in Fig. 6. The SDG moieties are esterified to each other via the linker molecule 3-hydroxy-3methyl glutaric acid (HMGA)(Klosterman and Smith, 1954). CoA-activated HMGA links to the C-6 of the glucosyl moieties of SDG resulting in dimer and higher oligomer formation (Ford et al., 2001). An average chain length of 5 SDG units has been suggested based on NMR analysis of the lignan macromolecule, resulting in an average molecular mass of 4000 (Kamal-Eldin et al., 2001). Besides SDG and HMGA, coumaric acid glucoside (CouAG) and ferulic acid glucoside (FeAG) have been identified as constituents of the lignan macromolecule (Ford et al., 2001; Johnsson et al., 2002).

In flaxseeds, lignans are mainly biosynthesized in the hulls (outer layer of the seeds) since expression of the PLR gene was the highest in that part of the seed (Hano et al., 2006). Besides, the hull fraction of the flaxseeds has been found to contain even higher amounts of lignans than whole seeds (Madhusudhan et al., 2000). For this reason, flaxseed hulls were used as lignan source in this study.


Figure 6. Chemical structure of the lignan macromolecule from flaxseed (Kamal-Eldin et al., 2001).

## Health effects of sesame seeds

The health effects of sesame seeds are often related to both the advantageous fatty acid composition and to the high concentration of antioxidants (Hemalatha et al., 2004), of which the lignans are important representatives. The antihypertensive effects of sesame seed lignans and their human metabolites are related to a down regulation of the nitric oxide metabolism resulting in a vasorelaxational effect (Lee et al., 2004; Nakano et al., 2006). Furthermore, hypocholesterolemic effects (Hirata et al., 1996; Wu et al., 2006) have been reported, as sesamin was suggested to inhibit cholesterol synthesis (HMG-CoA reductase) (Hirata et al., 1996). Lipid-lowering effects are reported as well for lignans from sesame seeds: hepatic fatty acid oxidation was increased and lipogenesis was decreased (Ide et al., 2003; Kiso, 2004). Under influence of sesamin, alcohol is metabolized faster and the
increase in face temperature after drinking alcohol is reduced (Kiso, 2004; Kiso et al., 2005). Finally, sesame seeds are suggested to have cancer preventive effects (Hirose et al., 1992; Jacklin et al., 2003).

## Lignans from sesame seeds

In Table 2, the average nutrient composition of sesame seeds is given. As for flaxseeds, relative high concentrations of lignans are present. However, the type of lignans differs from that in flaxseeds. In flaxseeds, mainly dibenzylbutane lignans are present, while in sesame seeds the methylenedioxybridged furanofuran lignans are predominant.




Figure 7. Chemical structures of aglyconic lignans from sesame seeds (Kamal-Eldin et al., 1994; Marchand et al., 1997; Moazzami et al., 2006b; Namiki, 2007; Osawa et al., 1985). Of sesaminol, sesamolinol and pinoresinol glucosylated forms have been described as well (Katsuzaki et al., 1994; Moazzami et al., 2006a).

Aglyconic lignans, mainly sesamin and sesamolin, are present in the oil fraction of sesame seeds (Dachtler et al., 2003; Kamal-Eldin and Appelqvist, 1994; Kamal-Eldin et al., 1994). Glucosylated lignans, as sesaminol glucosides, are mainly present in the defatted fraction of
sesame seeds (Moazzami et al., 2006a; Moazzami et al., 2006b; Shyu and Hwang, 2002; Suja et al., 2005). In Fig. 7 the chemical structures of lignans from sesame seeds are given. The exact lignan composition of sesame seeds changes with the developmental stage of the seeds (Jiao et al., 1998; Ono et al., 2006).

## Conversion of lignans by the human intestinal flora

Not the plant lignans, but the so-called mammalian lignans enterodiol (END) and enterolactone (ENL) are held responsible for the health effects related to lignan consumption. This is because in human plasma mainly mammalian lignans have been identified. The mammalian lignans are formed by a multi-step conversion reaction by the microflora of the human colon. The pathways leading from dibenzylbutane and methylenedioxybridged furanofuran lignans to the mammalian lignans will be discussed below.

## Fermentation of dibenzylbutane lignans

In 1980, enterodiol (END) and enterolactone (ENL) were identified in human urine and were called mammalian lignans (Setchell et al., 1980; Stitch et al., 1980). Because of the similarities in the cyclic pattern of excretion during the menstrual cycle of these mammalian lignans and estradiol, it was first suggested that they were formed in the uterus (Setchell et al., 1980). However, because the mammalian lignans were not produced in germ-free animals or subjects treated with antibiotics, it became clear that the mammalian lignans were formed via fermentation in the colon (Axelson and Setchell, 1981; Axelson et al., 1982; Setchell et al., 1981).
The fermentation of dibenzylbutane lignans has been found to consists of 4 steps: deglucosylation, demethylation, dehydroxylation and dehydrogenation. An overview of the pathway is shown in Fig. 8.

## Deglucosylation

The glycosidic lignans need to be deglucosylated prior to further conversion. Both in the small intestine and in the colon, brush border enzymes are present, which have the potential to deglycosylate phenolic compounds (Day et al., 1998; Lambert et al., 1999; Nemeth et al., 2003). However, no literature is available on the action of these enzymes on lignans.

From human feces, several bacteria were isolated, which are able to form the lignan aglycons. The capability to deglucosylate is widespread over the bacteria, but especially members of the Bacteroides, one of the most dominant groups of the fecal flora (Zoetendal et al., 2006), are good deglucosylating bacteria (Clavel et al., 2006b). Also several members of the Clostridia are able to deglucosylate lignans (Clavel et al., 2006a; Clavel et al., 2007).

## Demethylation

As the second step in the conversion of the plant lignans into the mammalian lignans, the methyl groups are being removed from the methoxyl moieties of the aglyconic lignans. Bacteroides methylotrophicum, Eubacterium callanderi, Eubacterium limosum and

Peptostreptococcus productus have been identified as bacteria capable of demethylating SECO (Clavel et al., 2006b; Wang et al., 2000).


Figure 8. Fermentation pathway from SDG to the mammalian lignans (adapted from (Wang et al., 2000)).

These bacteria belong to the group of acetogenic bacteria. They are found in almost all anaerobic environments, including the colon (Drake et al., 2002; Lajoie et al., 1988). Acetogenic bacteria have the ability to convert one-carbon sources, as $\mathrm{CO}_{2}$ or methyl groups via the acetyl-CoA pathway into acetate for energy metabolism and biosynthesis, as shown in Fig. 9A (Diekert, 1992; Drake et al., 2002; Muller et al., 2004). $\mathrm{CO}_{2}$ is both the carbon source and the final electron acceptor and $\mathrm{H}_{2}$ functions as electron donor. Methyl groups act both as carbon source and electron donor. The $O$-demethylases transfer the methyl moiety from a methoxyl group to tetrahydrofolate (Fig. 9B, first reaction). Tetrahydrofolate enters the acetyl-CoA pathway. Via the reversed methyl branch of the acetyl-CoA pathway one methylated tetrahydrofolate is oxidized, and one $\mathrm{CO}_{2}$, six electrons and six hydrogens are generated (Fig. 9B, second reaction). These products enter the carbonyl branch of the acetyl-CoA pathway together with two exogenous $\mathrm{CO}_{2}$. As a result three CO intermediates are formed, which, together with three methylated tetrahydrofolates from the methyl branch, enter the final part of the acetyl-CoA pathway, yielding three molecules of acetyl-CoA (Muller et al., 2004). Since this is a general mechanism for the conservation of energy of bacteria growing under anaerobic conditions, a large number of bacteria is able to demethylate.

Chapter 1
A

Carbonyl branch
$\mathrm{CO}_{2}$

B
$4 \mathrm{R}-\mathrm{O}-\mathrm{CH}_{3}$
$\xrightarrow{\text { O-demethylase }} \rightarrow 4 \mathrm{R}-\mathrm{OH}+4 \mathrm{CH}_{3}-\mathrm{H}_{4} \mathrm{~F}$
inversed methyl branch
$1 \mathrm{CH}_{3}-\mathrm{H}_{4} \mathrm{~F}+\mathrm{H}_{2} \mathrm{O}$
acetyl-CoA pathway $\rightarrow \mathrm{CO}_{2}+6 \mathrm{H}^{+}+6 \mathrm{e}$
methyl branch acetyl-
$3 \mathrm{CH}_{3}-\mathrm{H}_{4} \mathrm{~F} \quad$ corrinoid enzyme $\xrightarrow{\text { CoA pathway }} \rightarrow 3 \mathrm{CH}_{3}$-corrinoid enzyme
carbonyl branch acetyl-
$3 \mathrm{CO}_{2}+6 \mathrm{H}^{+}+6 \mathrm{e}^{-}$
CoA pathway $\ldots-\cdots 3 \mathrm{CO}+3 \mathrm{H}_{2} \mathrm{O}$
$3 \mathrm{CO}+3 \mathrm{CH}_{3}$-corrinoid enzyme $\underset{\text { corrinoid enzyme }}{\mathrm{CoA}} 3$ acetyl-CoA
In total:
$4 \mathrm{R}-\mathrm{O}-\mathrm{CH}_{3}+2 \mathrm{CO}_{2} \quad$ acetyl-CoA pathway $\rightarrow 4 \mathrm{R}-\mathrm{OH}+3$ acetyl-CoA $+2 \mathrm{H}_{2} \mathrm{O}$

Figure 9. (A) Schematic representation of the Acetyl-CoA pathway. (B) Reactions describing the conversion of methyl groups into acetyl-CoA via the (inversed) acetyl-CoA pathway (adapted from (Muller et al., 2004)).

## Dehydroxylation

The third step in the conversion of SECO to the mammalian lignans is the dehydroxylation step. During this reaction, the hydroxyl group at the para-position of the phenolic ring is cleaved off. Until now, Eggerthella lenta (previously called Eubacterium lentum (Kageyama et al., 1999)) is the only species identified able to dehydroxylate previously demethylated lignans (Jin et al., 2007b; Wang et al., 2000). These baceteria work in an enantioselective way. Dependent on the strain isolated (+)- or (-)-demethylated SECO was converted into (+)- or (-)-END, respectively (Jin et al., 2007b). In addition, Clostridium scindens in co-incubation with Peptostreptococcus productus can ferment SECO into END (Clavel et al., 2006b).

## Dehydrogenation

The final step in the fermentation is the conversion of END into ENL. Clostridium amygdalinium and Clostridium saccharolyticum are examples of strains capable to dehydrogenate END (Clavel et al., 2006b). On the contrary, these strains were not capable of dehydrogenating SECO. Furthermore, Lactonifactor longoviformis and a Ruminococcus strain are able to oxidize END to ENL (Clavel et al., 2007; Jin et al., 2007a). In line with the dehydroxylation step, also the dehydrogenation is an enantioselective reaction, although bacteria catalyzing conversions of both isoforms have been isolated from the human feces (Jin et al., 2007a).

## Fermentation of methylenedioxybridged furanofuran lignans

For a long time SECO was thought to be the main precursor of mammalian lignans. However, upon consumption of sesame seeds, ENL and END levels in human urine increased in a similar way as after consumption of flaxseed (Coulman et al., 2005). Thus, also the methylenedioxybridged lignans have been suggested to be precursors of ENL and END.
Fermentation of methylenedioxybridged furanofuran lignans should involve, besides the steps described for dibenzylbutane lignans, a reductive cleavage of the furanofuran rings and oxidative demethylation of the methylenedioxybridges.
In order to isolate bacteria involved in these reaction, sesamin has been incubated with a human fecal slurry. These studies showed, however, that only $1 \%$ of the sesamin was converted into mammalian lignan (Liu et al., 2006). Since in in vivo studies mammalian lignans were formed in larger quantities (Liu et al., 2006; Penalvo et al., 2005), it has been suggested that endogenous metabolism is involved in the conversion reaction of methylenedioxybridged lignans to mammalian lignans.
It was shown that intact sesamin can be identified in several tissues, especially in the liver (Umeda-Sawada et al., 1999). These high concentrations in the liver suggest that liver metabolism plays a role. Involvement of metabolism by liver enzymes was confirmed by a study, which showed that liver microsomes produce metabolites with cleaved methylenedioxybridges (Nakai et al., 2003). These intermediates are thought to be excreted in the colon via the enterohepatic circulation. The intermediates are further fermented into mammalian lignans in the colon (Liu et al., 2006; Nakai et al., 2003; Penalvo et al., 2005). The putative conversion pathway of the methylenedioxybridged furanofuran lignans is shown in Fig. 10. The actual order of formation of the intermediates is currently unknown.


Figure 10. Putative conversion pathway of the methylenedioxybridged furanofuran lignan sesamin into the mammalian lignans. $\mathrm{R}=\mathrm{H}$ or $\mathrm{CH}_{3}$. In the liver the methylenedioxybridges are cleaved resulting in either a catechol moiety $(\mathrm{R}=\mathrm{H})$ or a methoxyl moiety $\left(\mathrm{R}=\mathrm{CH}_{3}\right)$. The cleavage of the furan rings most likely takes place in the colon. The exact order of the reactions is unknown.

## Estrogen metabolism

Lignans are thought to have estrogenic properties because of their structural similarities with human estradiol and synthetic estrogens (Adlercreutz et al., 1987; Setchell et al., 1981). Lignans can interact with the estrogen metabolism at different levels. They are suggested to influence the estrogen receptor-mediated gene transcription, to influence human estradiol biosynthesis and/or to have an effect on the estrogen transport mechanism. First, the human estrogen metabolism is discussed followed by how lignans can interfere with this metabolism.

## Biosynthesis of $17 \beta$-estradiol

$17 \beta$-estradiol (or simply estradiol) is the most potent member of the human estrogens. Estrogens are a class of steroid hormones, which are, among others, in control of the female sex organs and the reproduction. They are formed in the female ovaries and in peripheral tissue of both man and women.
Estradiol is biosynthesized from cholesterol. This pathway is summarized in Fig. 11. The first step in this biosynthesis pathway is the conversion of cholesterol into pregnenolone. Via progesterone or via 17-hydroxy-pregenenolone, androstenedione is formed. Androstenedione is, under influence of $17 \beta$-hydroxysteroid dehydrogenase, in equilibrium with testosterone. Testosterone is, by the action of aromatase, directly converted to


Figure 11. Biosynthetic pathway of $17 \beta$-estradiol (Janer and Porte, 2007).
estradiol. Androstenedione is first converted to estrone by the action of aromatase, and further into estradiol by the action of $17 \beta$-hydroxysteroid dehydroxygenase (Korach et al., 1995).

After formation, estrogens are secreted into the blood. They are transported though the human body by binding to carrier proteins as albumins or globulins (mainly sex hormone binding globulin; SHBG). A few percent of the estradiol is present in an unbound form (Korach et al., 1995).

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## The estrogen receptor

The estrogen receptor (ER) belongs to the nuclear hormone receptor superfamily and functions in a similar way as all members of this superfamily. Of the estrogen receptor, two isoforms have been described: ER $\alpha$ and ER $\beta$ (Mosselman et al., 1996). They both contain 6 distinct regions (A-F; see Fig. 12). The A/B-region is positioned at the N -terminus. It contains one of the activation factors (AF-1), which are important for the regulation of transcription. The A/B-region of ER $\beta$ is shorter than that of the ER $\alpha$ and, therefore, the ER $\beta$ does not contain a fully active AF-1 (Hall and McDonnell, 1999). The C-region is the highly conserved DNA binding domain (DBD). The fact that this region is highly conserved indicates that both receptors can bind to the same estrogen responsive elements of the DNA. The E/F-region contains the ligand binding domain (LBD). This domain is less well conserved compared to the DBD and, therefore, the affinity of both receptors for different ligands varies. The LBD also contains the second activation factor (AF-2) (Lonard and Smith, 2002; Muramatsu and Inoue, 2000). The LBD is folded into a wedge-shaped sizeable scaffold, which allows ligand binding (Brzozowski et al., 1997). Upon ligand binding, the orientation of helix 12, one of the structural elements of the LBD, changes. The ER $\alpha$ and ER $\beta$ are differently distributed over the various tissues. Often both types of estrogen receptors are present but one of the two is predominating. ER $\alpha$ is predominant in the testis, uterus, testis, pituitary gland, kidney and adrenal glands, while ER $\beta$ is predominating in the ovaries and the prostate (Kuiper et al., 1997). Besides direct responses via gene expression, ER $\beta$ has been suggested to play a role as modulator of ER $\alpha$ activity (Hall and McDonnell, 1999).


Figure 12. Schematic representation of $\mathrm{ER} \alpha$ and $\mathrm{ER} \beta$. $\mathrm{ER} \alpha$ is 595 amino acids long, ER $\beta 530$ amino acids. The DNA binding domain (DBD) shows high homology between ER $\alpha$ and ER $\beta$. The ligand binding domain (LBD) is less well conserved. The percentages indicate the amino acid homology (Lonard and Smith, 2002).

## Estrogen receptor induced gene expression

When ligands bind to the ER, a cascade of reactions is induced, which finally results in the transcription of target genes as depicted in Fig. 13. The final effect is dependent on the type of ligand, the structure of the ligand, and the kind of co-activator recruited.
Without the presence of a ligand, a basal level of gene transcription is observed (Fig. 13A). It is suggested that unliganded ER forms dimers, which slightly interact with the estrogen responsive element (ERE) resulting in a basal level of gene transcription (Hall and McDonnell, 1999; Klinge, 2001; Korach et al., 1995).

When an ER-agonist binds to the ER (Fig. 13B), in particular the helix 12 undergoes a conformational change, dimerisation takes place and the liganded ER-dimer binds to the ERE. Ligand binding stabilizes ER-ERE interaction. The liganded ERE-bound ER-dimer recruits co-activators (Korach et al., 1995). The orientation of the helix 12, cell-type and ER type determine which co-activators can be recruited (Hall and McDonnell, 1999; Mueller et al., 2004). Consecutive association of several other transcription factors leads to induction of gene transcription (Korach et al., 1995).
The basal level of gene transcription is deminished after binding of an ER antagonist (Hall and McDonnell, 1999). Upon antagonist binding, helix 12 folds differently than upon agonist binding (Fig. 13C). This shape prevents the interaction of the ER-dimer-antagonist with the ERE and induces the degradation of ER (Jordan, 1995).
Ligand

binding \begin{tabular}{c}
Dimerisation <br>
of two ER

 

Binding of dimerized <br>
ER to ERE

$\quad$

Co-activator <br>
recruitment

$\quad$

Co-activator <br>
recruitment induces
\end{tabular}

A: without ligand







Figure 13. Activation of gene expression via ER. ERE = estrogen responsive element; SERM = selective estrogen receptor modulator. A: In the absence of a ligand, a basal level of transcription takes place. B: Upon binding of an agonist, several co-activators can bind resulting in the transcription of several genes. C: Upon antagonist binding, antagonist-ER complex can not bind to ERE. D: Depending on the kind of SERM, only some co-activators can be recruited resulting in the transcription of a limited number of genes. Upon recruitment of a co-repressor, transcription can not take place.

Many phytoestrogens have been reported to be able to behave both as an agonist or as an antagonist dependent on the structure of the compound, the cell type and the types of coactivators present (Kuiper et al., 1997; Lonard and Smith, 2002; Mueller et al., 2004). These compounds are called selective estrogen receptor modulators (SERM).
When a SERM binds to the ER, helix 12 folds in a particular way, which largely determines the mode of action of the SERM (Lonard and Smith, 2002; Pike et al., 1999).
SERM binding leads to a helix 12 orientation intermediate between that of an agonist and antagonist (Fig. 13D)(Lonard and Smith, 2002), dimerisation and ERE binding can take place, but only some of the co-activators can bind. The type of co-activator recruited as a result of SERM binding determines, which genes are induced. Since the type of coactivators differ per cell type, a SERM can have agonistic effects in a certain cell type, while in another cell type it has antagonistic effects.
Furthermore, so-called co-repressors also play a role in determining whether a SERM functions as a agonist or antagonist (Lonard and Smith, 2002). Co-repressors prevent the induction of gene expression, but the exact physiological role is unclear. Probably, they prevent certain co-activators from binding and in that way, they are involved in determining the nature of a SERM (Lonard and Smith, 2002)

## Estrogenic effects of lignans

As mentioned before, lignans can influence estrogen metabolism by three different mechanisms: by interaction with ER-mediated gene transcription, by influencing estrogen transport and by influencing estradiol biosynthesis. An overview of the studies on the estrogenicity of lignans is shown in Table 3.

## Estrogenic effects of lignans by interaction with the estrogen receptor

Lignans can bind to the estrogen receptor, which induces the ER-mediated gene transcription cascade. Besides, lignans might also interact with other steps of this cascade, for example, with co-activator binding.
Various protocols have been used to study the effect of lignans on the ER-mediated transcription pathway, as mentioned in Table 3. Displacement assays are regularly used to investigate the interaction of lignans with the ER. Effects on gene transcription in transfected cell lines are often studied to investigate the complete cascade. Furthermore, effects on cell proliferation, which is an in vitro marker for ER-mediated effects (Carreau et al., 2008), and in vivo effects on estrogen target tissues as uterus, vagina and bone, are studied (Penttinen et al., 2007).

The mammalian lignans END and ENL, and the liver metabolite 6OH-ENL, have been studied for their interaction with the ER-mediated gene transcription cascade. However, the results of the different studies are inconsistent and incomplete.
ENL showed weak interactions with the human ER $\alpha$ and ER $\beta$ compared to estradiol in displacement assays although the order of potency between two types of ER differed. Mueller et al. (2004) reported a stronger affinity for the ER $\alpha$ than for ER $\beta$, while Pentinnen
et al. (2007) reported a stronger interaction with ER $\beta$ than with $\mathrm{ER} \alpha$ (see Table 3 for $\mathrm{IC}_{50}$ values). Furthermore, ENL seems to induce more gene transcription via ER $\alpha$ than via ER $\beta$ (Carreau et al., 2008; Penttinen et al., 2007).
END has not been studied for its ER-binding. In a study investigating gene transcription, END was shown to be a more potent estrogen and acted more similar as estradiol compared to ENL (Carreau et al., 2008).
Since non of the intermediate products of the bioconversion have been tested, no information is available on their interaction with this cascade.

In general, the concentrations at which effects on gene transcription have been observed, were lower than expected based on those found for binding to the ER. Therefore, metabolism of ENL by the cell system was suggested to take place, converting the mammalian lignans into more estrogenic compounds (Penttinen et al., 2007). This conversion is probably cell type specific.

## Estrogenic effects of lignans via SHBG

Sex hormone binding globulin (SHBG) is, together with some albumins, responsible for the transport of estrogens and other hormones through the human body. Binding of lignans to SHBG influences estradiol distribution through the human body. Besides, SHBG interacts with receptors of sex hormone target tissues as the prostate (Hryb et al., 1985). This interaction only takes place when a substrate is bound to the binding site of SHBG. This suggests that all compounds binding to SHBG can exert an effect on the sex hormone target tissues.
Lignans have been reported to bind to SHBG and to influence its biosynthesis (Adlercreutz et al., 1987; Hillerns et al., 2005; Schöttner et al., 1997).
The results as shown in Table 3 seem to indicate that bioconversion of SECO into the mammalian lignans reduces SHBG-binding affinity. A similar trend has been observed for the bioconversion of a dehydrated form of SECO, anhydrosecoisolariciresinol (AHS), into the dehydrated counterpart of END, enterofuran (ENF) (Schöttner et al., 1997). Since these are the results of only one study, they need to be confirmed before definite conclusions can be drawn.

## Effects of lignans on estradiol biosynthesis

Lignans have been shown to interact with estradiol biosynthesis (Fig. 11). They have been shown to inhibit aromatase and $17 \beta$-hydroxysteroid dehydrogenase ( $17 \beta$-HSD) activity (Brooks and Thompson, 2005; Xu et al., 1994), resulting in a lower circulating concentration of human estrogens.
ENL and END have been shown to inhibit aromatase (Adlercreutz et al., 1993; Brooks and Thompson, 2005; Lacey et al., 2005; Xu et al., 1994). In general, ENL showed stronger inhibition of aromatase activity compared to END. The presence of the lactone ring in ENL is suggested to increase the affinity to aromatase or to increase the lipid solubility, resulting in better uptake in the cells. As a consequence of a higher uptake, aromatase is exposed to higher concentrations ENL resulting in more inhibition (Mäkelä et al., 2000; Wang et al., 1994).
Tabel 3. Overview of estrogenic effects of lignans reported in literature.


Only one study investigated the aromatase inhibition of two of the intermediates of the bioconversion of SECO to END. This study showed no effects of mono-demethylated SECO (MDEND) and di-demethylated SECO (HDEND) on aromatase activity. So far, plant lignans have not been tested for their influence on estradiol biosynthesis.

Since only ENL was tested for all three mechanisms, and because of the variability between the assays, it is unclear which of the three mechanisms (interaction with the ER-mediated gene transcription cascade, influence on SHBG or influence on estradiol biosynthesis) is the most important. Furthermore, it is unclear how the structure of lignans influences their functionality. Particularly for the microbial conversion products it is largely unknown how structural modifications affect estrogenicity.

## Antioxidant activity of lignans

Interaction with human estrogen metabolism is not the only mechanism by which lignans can exert effects on human metabolism. They are also reported to have antioxidant activity. An overview of the antioxidant activity of lignans is given in Table 4. In the upper part of the table the dibenzylbutane lignans are listed. In the second part of the table, the furanofuran lignans are described.
The results of the different studies are hard to compare with each other, because different kinds of assays and reference compounds were used. This makes it hard to conclude, which structural elements determine antioxidant activity. Glucosylation of lignans has been shown to reduce the antioxidant activity (Hosseinian et al., 2007; Miyake et al., 2005). Furthermore, the results of the different studies are not always consistent, which makes it even more difficult to draw conclusions on structure-function relationships.
The antioxidant activity of the intermediates formed during fermentation has not been investigated at all. Since some of these intermediates have a catechol moiety, their antioxidant activity might be enhanced compared to plant lignans.

The good antioxidant properties of lignans as SECO and SDG can be explained by the observation that radicals can be neutralized by dimerization. This regenerates the antioxidative potential of the lignans (Eklund et al., 2005; Hosseinian et al., 2007). Furthermore, synergistic effects of lignans and tocopherols with respect to their antioxidative effects (Chung et al., 2006; Ghafoorunissa et al., 2004), are probably due to the recycling of the tocopherols (Ghafoorunissa et al., 2004).
No prooxidant activity is observed for SDG, END or ENL (Kitts et al., 1999) and also the polymerized reaction products are stable and will not result in a prooxidant effect (Hosseinian et al., 2007).

The main lignans in sesame oil, sesamin and sesaminol hardly possess antioxidant activities. Therefore, the antioxidant activity of sesame oil have been attributed to sesamol and not to sesamin and sesamolin. Sesamol (3,4-methylenedioxyphenol), technically

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Table 4. Antioxidant activity of lignans.

spoken not a lignan, is a degradation product of sesamolin. During frying/roasting (heating above $150{ }^{\circ} \mathrm{C}$ ), but also during in vivo metabolism, sesamolin is converted into sesamol and/or sesaminol (Fukuda et al., 1994; Kang et al., 1998). Sesamol has been shown to be a better oxidant than $\alpha$-tocopherol and BHT (Lee and Choe, 2006; Suja et al., 2004; Suja et al., 2005).

Besides the estrogenic and antioxidant activities of lignans, there are several other mechanisms, which might explain the health effects of lignans. Some examples of other mechanisms are interaction with other receptors like the PXR-receptor (Jacobs et al., 2005), inhibition of enzyme expression, for example the enzymes involved in blood pressure regulation (Lee et al., 2004), or inhibition of particular enzyme activity (Jungestrom et al., 2007; Penumathsa et al., 2007). However, because these mechanisms are not extensively studied, they are not discussed further.

## Aims and outline of this thesis

Despite the fact that the composition of the lignan macromolecule from flaxseed has been investigated, its structure is largely unknown. Furthermore, it is known that lignans are converted into the bioactive mammalian lignans in the colon, but protocols to produce these lignans by single strain fermentations are sparsely available as are efficient protocols for their recovery from fermentation cultures. In order to address these points, the aims of the research described in this thesis are to identify the precise composition and structure of the lignan macromolecule from flaxseeds, to convert plant lignans into the mammalian lignans by fermentation, and to investigate how the bioconversion of lignans influences their estrogenicity. In order to be able to reach these goals, analytical and preparative protocols need to be developed.

Sesame seeds and flaxseeds were used as lignan sources. In flaxseeds, dibenzylbutane lignans are present, linked within a macromolecular structure. The composition and structure of this lignan macromolecule was investigated. In chapter 2 , the main constituents of the lignan macromolecule, including a novel constituent, are being described. Chapter 3 demonstrates how the various constituents are linked to each other. In chapter 4, the constituents of the lignan macromolecule were quantified, the variation in chain length was studied, and the relationship between the size and the composition of the lignan macromolecule is described.

In chapter 5, the fermentation of lignans from flaxseeds into the mammalian lignans and the identification of their intermediates are described. By treating the lignans from flaxseeds with acid, a furan ring is formed in the aliphatic part of the molecule. Fermentation of acidtreated lignans resulted in a similar array of intermediates as for the lignans from flaxseed. The preparation and fermentation of these acid-treated lignans are also described in chapter 5.

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Sesame seeds were used as a source of methylenedioxybridged furanofuran lignans. The conversion of these lignans to mammalian lignans was investigated. However, a different MS protocol was needed than used in chapters 2-5. A new MS protocol for the identification of all lignans in (fermented) extracts of sesame seeds, is described in chapter 6. In chapter 7 all results are discussed and some considerations on the applicability of the results are given.

## References

Adlercreutz, H., Höckerstedt, K., Bannwart, C., Bloigu, S., Hämäläinen, E., Fotsis, T., Ollus, A., 1987. Effect of dietary components, including lignans and phytoestrogens, on enterohepatic circulation and liver metabolism of estrogens and on sex hormone binding globulin (SHBG). J. Steroid Biochem. 27, 1135-1144.

Adlercreutz, H., Bannwart, C., Wähälä, K., Mäkelä, T., Brunow, G., Hase, T., Arosemena, P.J., Kellis, J.T.J., Vickery, L.E., 1993. Inhibition of human aromatase by mammalian lignans and isoflavonoid phytoestrogens. J. Steroid. Biochem. Mol. Biol. 44, 147-153.

Adlercreutz, H., 2002. Phytoestrogens and breast cancer. J. Steroid Biochem. Mol. Biol. 83, 113-118.

Andlauer, W., Furst, P., 2002. Nutraceuticals: a piece of history, present status and outlook. Food Res. Int. 35, 171-176.

Axelson, M., Setchell, K.D.R., 1981. The excretion of lignans in rats - evidence for an intestinal bacterial source for this new group of compounds. FEBS Lett. 123, 337-342.

Axelson, M., Sjövall, J., Gustafsson, B.E., Setchell, K.D.R., 1982. Origin of lignans in mammals and identification of a precursor from plants. Nature 298, 659-660.

Bakke, J.E., Klosterman, H.J., 1956. A new diglucoside from flaxseed. Proc. North Dakota Acad. Sci. 10, 18-21.

Boerjan, W., Ralph, J., Baucher, M., 2003. Lignin biosynthesis. Annu. Rev. Plant Biol. 54, 519-546.

Bommareddy, A., Arasada, B.L., Mathees, D.P., Dwivedi, C., 2006. Chemopreventive effects of dietary flaxseed on colon tumor development. Nutr. Cancer 54, 216-222.

Brooks, J.D., Thompson, L.U., 2005. Mammalian lignans and genistein decrease the activities of aromatase and 17 beta-hydroxy steroid dehydrogenase in MCF-7 cells. J. Steroid Biochem. Mol. Biol. 94, 461-467.

Brzozowski, A.M., Pike, A.C.W., Dauter, Z., Hubbard, R.E., Bonn, T., Engström, O., Öhman, L., Greene, G.L., Gustafsson, J.Â., Carlquist, M., 1997. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389, 753-758.

Carreau, C., Flouriot, G., Bennetau-Pelissero, C., Potier, M., 2008. Enterodiol and enterolactone, two major diet-derived polyphenol metabolites have different impact on ERalpha transcriptional activation in human breast cancer cells. J. Steroid Biochem. Mol. Biol. 110, 176-185.

Chen, J., Power, K.A., Mann, J., Cheng, A., Thompson, L.U., 2007. Flaxseed alone or in combination with tamoxifen inhibits MCF-7 breast tumor growth in ovariectomized Athymic mice with high circulating levels of estrogen. Exp. Biol. Med. 232, 1071-1080.

Chen, J.M., Stavro, P.M., Thompson, L.U., 2002. Dietary flaxseed inhibits human breast cancer growth and metastasis and downregulates expression of insulin-like growth factor and epidermal growth factor receptor. Nutr. Cancer 43, 187-192.

Chen, J.M., Tan, K.P., Ward, W.E., Thompson, L.U., 2003. Exposure to flaxseed or its purified lignan during suckling inhibits chemically induced rat mammary tumorigenesis. Experim. Biol. Med. 228, 951-958.

Chen, J.M., Hui, E., Ip, T., Thompson, L.U., 2004. Dietary flaxseed enhances the inhibitory effect of tamoxifen on the growth of estrogen-dependent human breast cancer (MCF-7) in nude mice. Clin. Cancer Res. 10, 7703-7711.

Chung, J., Lee, Y., Choe, E., 2006. Effects of sesame oil addition to soybean oil during frying on the lipid oxidative stability and antioxidants contents of the fried products during storage in the dark. J. Food Sci. 71, C222-C226.

Clavel, T., Borrmann, D., Braune, A., Dore, J., Blaut, M., 2006a. Occurrence and activity of human intestinal bacteria involved in the conversion of dietary lignans. Anaerobe 12, 140147.

Clavel, T., Henderson, G., Engst, W., Dore, J., Blaut, M., 2006b. Phylogeny of human intestinal bacteria that activate the dietary lignan secoisolariciresinol diglucoside. FEMS Microbiol. Ecol. 55, 471-478.

Clavel, T., Lippman, R., Gavini, F., Dore, J., Blaut, M., 2007. Clostridium saccharogumia sp nov and Lactonifactor longoviformis gen. nov., sp nov., two novel human faecal bacteria involved in the conversion of the dietary phytoestrogen secoisolariciresinol diglucoside. Syst. Appl. Microbiol. 30, 16-26.

Cosentino, M., Marino, F., Ferrari, M., Rasini, E., Bombelli, R., Luini, A., Legnaro, M., Delle Canne, M.G., Luzzani, M., Crema, F., Paracchini, S., Lecchini, S., 2007. Estrogenic activity of 7-hydroxymatairesinol potassium acetate (HMR/lignan (TM)) from Norway spruce (Picea abies) knots and of its active metabolite enterolactone in MCF-7 cells. Pharmacol. Res. 56, 140-147.

Costa, M.A., Collins, R.E., Anterola, A.M., Cochrane, F.C., Davin, L.B., Lewis, N.G., 2003. An in silico assessment of gene function and organization of the phenylpropanoid pathway metabolic networks in Arabidopsis thaliana and limitations thereof.
Phytochemsitry 64, 1097-1112.

Coulman, K.D., Liu, Z., Hum, W.Q., Michaelides, J., Thompson, L.U., 2005. Whole sesame seed is as rich a source of mammalian lignan precursors as whole flaxseed. Nutr. Canc. Int. J. 52, 156-165.

Dabrowski, K.J., Sosulski, F.W., 1984. Composition of free and hydrolyzable phenolic acids in defatted flours of ten oilseeds. J. Agric. Food Chem. 32, 128-130.

Dachtler, M., van de Put, F.H.M., von Stijn, F., Beindorff, C.M., Fritsche, J., 2003. On-line LC-NMR-MS characterization of sesame oil extracts and assessment of their antioxidant activity. Eur. J. Lipid Sci. Technol. 105, 488-496.

Davin, L.B., Lewis, N.G., 1992. Phenylpropanoid metabolism: Biosynthesis of monolignols, lignans and neolignans, lignins and suberins. In: Stafford, H.A. (Eds.), Phenolic metabolism in Plants. Plenum Publisher Corporation, Washington, pp. 325-375.

Davin, L.B., Wang, H.B., Crowell, A.L., Bedgar, D.L., Martin, D.M., Sarkanen, S., Lewis, N.G., 1997. Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. Science 275, 362-366.

Davin, L.B., Lewis, N.G., 2000. Dirigent proteins and dirigent sites explain the mystery of specificity of radical precursor coupling in lignan and lignin biosynthesis. Plant Physiol. 123, 453-461.

Davin, L.B., Lewis, N.G., 2005. Dirigent phenoxy radical coupling: advances and challenges. Curr. Opin. Biotechnol. 16, 398-406.

Day, A.J., DuPont, M.S., Ridley, S., Rhodes, M., Rhodes, M.J.C., Morgan, M.R.A., Williamson, G., 1998. Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver beta-glucosidase activity. FEBS Lett. 436, 71-75.

Demark-Wahnefried, W., Robertson, C.N., Walther, P.J., Polascik, T.J., Paulson, D.F., Vollmer, R.T., 2004. Pilot study to explore effects of low-fat, flaxseed-supplemented diet on proliferation of benign prostatic epithelium and prostate-specific antigen. Urology 63, 900-904.

Diekert, G., 1992. The Acetogenic Bacteria. In: Balows, A., Truper, H.G., Dworkin, M., Harder, W., Schleifer, K.H. (Eds.), The Prokaryotes. Spinger-Verlag, New York, pp. 517533.

Dinkova-Kostova, A.T., Gang, D.R., Davin, L.B., Bedgar, D.L., Chu, A., Lewis, N.G., 1996. (+)-Pinoresinol/(+)-lariciresinol reductase from Forsythia intermedia - Protein purification, cDNA cloning, heterologous expression and comparison to isoflavone reductase. J. Biol. Chem. 271, 29473-29482.

Drake, H.L., Kusel, K., Matthies, C., 2002. Ecological consequences of the phylogenetic and physiological diversities of acetogens. Antonie Van Leeuwenhoek 81, 203-213.

Eklund, P.C., Langvik, O.K., Warna, J.P., Salmi, T.O., Willfor, S.M., Sjoholm, R.E., 2005. Chemical studies on antioxidant mechanisms and free radical scavenging properties of lignans. Org. Biomol. Chem. 3, 3336-3347.

Ford, J.D., Huang, K.S., Wang, H.B., Davin, L.B., Lewis, N.G., 2001. Biosynthetic pathway to the cancer chemopreventive secoisolariciresinol diglucoside-hydroxymethyl glutaryl ester- linked lignan oligomers in flax (Linum usitatissimum) seed. J. Nat. Prod. 64, 1388-1397.

Fukuda, Y., Osawa, T., Kawakishi, S., Namiki, M., 1994. Chemistry of lignan antioxidants in sesame seed and oil. In: Ho, C.T., Osawa, T., Huang, M.T., Rosen, R.T. (Eds.), Food phytochemicals for cancer prevention II: Teas, spices and herbs. American chemical society, Washington, pp. 264-274.

Gang, D.R., Costa, M.A., Fujita, M., Dinkova-Kostova, A.T., Wang, H.B., Burlat, V., Martin, W., Sarkanen, S., Davin, L.B., Lewis, N.G., 1999. Regiochemical control of monolignol radical coupling: a now paradigm for lignin and lignan biosynthesis. Chem. Biol. 6, 143-151.

Ghafoorunissa, Hemalatha, S., Rao, M.V.V., 2004. Sesame lignans enhance antioxidant activity of vitamin E in lipid peroxidation systems. Mol. Cell. Biochem. 262, 195-202.

Hall, C.A., Tulbek, M.C., Xu, Y., 2006. Flaxseed. In: Taylor, S. (Eds.), Advances in food and nutrition research. Elsevier Inc., San Diego, pp. 1-97.

Hall, J.M., McDonnell, D.P., 1999. The estrogen receptor beta-isoform (ER beta) of the human estrogen receptor modulates ER alpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology 140, 5566-5578.

Halls, S.C., Davin, L.B., Kramer, D.M., Lewis, N.G., 2004. Kinetic study of coniferyl alcohol radical binding to the (+)-pinoresinol forming dirigent protein. Biochem. 43, 25872595.

Hallund, J., Tetens, I., Bugel, S., Tholstrup, T., Ferrari, M., Teerlink, T., Kjaer, A., Wiinberg, N., 2006. Daily consumption for six weeks of a lignan complex isolated from flaxseed does not affect endothelial function in healthy postmenopausal women. J. Nutr. 136, 2314-2318

Hano, C., Martin, I., Fliniaux, O., Legrand, B., Gutierrez, L., Arroo, R.R.J., Mesnard, F., Lamblin, F., Laine, E., 2006. Pinoresinol-lariciresinol reductase gene expression and secoisolariciresinol diglucoside accumulation in developing flax (Linum usitatissimum) seeds. Planta 224, 1291-1301.

Hardy, G., 2000. Nutraceuticals and functional foods: Introduction and meaning. Nutrition 16, 688-689.

Hemalatha, S., Raghunath, A., Ghafoorunissa, 2004. Dietary sesame (Sesamum indicum cultivar Linn) oil inhibits iron-induced oxidative stress in rats. Br. J. Nutr. 92, 581-587.

Hemmati, S., Schmidt, T.J., Fuss, E., 2007. (+)-Pinoresinol/(-)-lariciresinol reductase from Linum perenne Himmelszelt involved in the biosynthesis of justicidin B. FEBS Lett. 581, 603-610.

Hillerns, P.I., Zu, Y.G., Fu, Y.J., Wink, M., 2005. Binding of phytoestrogens to rat uterine estrogen receptors and human sex hormone-binding globulins. Z. Naturforsch.(C) 60, 649656.

Hirata, F., Fujita, K., Ishikura, Y., Hosoda, K., Ishikawa, T., Nakamura, H., 1996. Hypocholesterolemic effect of sesame lignan in humans. Atherosclerosis 122, 135-136.

Hirose, N., Doi, F., Ueki, T., Akazawa, K., Chijiiwa, K., Sugano, M., Akimoto, K., Shimizu, S., Yamada, H., 1992. Suppressive Effect of Sesamin against 7,12-Dimethylbenz a -Anthracene Induced Rat Mammary Carcinogenesis. Anticancer Res. 12, 1259-1266.

Hosseinian, F.S., Muir, A.D., Westcott, N.D., Krol, E.S., 2006. Antioxidant capacity of flaxseed lignans in two model systems. J. Am. Oil Chem. Soc. 83, 835-840.

Hosseinian, F.S., Muir, A.D., Westcott, N.D., Krol, E.S., 2007. AAPH-mediated antioxidant reactions of secoisolariciresinol and SDG. Org. Biomol. Chem. 5, 644-654.

Hryb, D.J., Khan, M.S., Rosner, W., 1985. Testosterone-Estradiol-Binding Globulin binds to human prostatic cell-membranes. Biochem. Biophys. Res. Commun. 128, 432-440.

Ide, T., Kushiro, M., Takahashi, Y., Shinohara, K., Fukuda, N., Sirato-Yasumoto, S., 2003. Sesamin, a sesame lignan, as a potent serum lipid-lowering food component. Jpn. Agric. Res. Q. 37, 151-158.

Jacklin, A., Ratledge, C., Welham, K., Bilko, D., Newton, C.J., 2003. The sesame seed oil constituent, sesamol, induces growth arrest and apoptosis of cancer and cardiovascular cells. In: (Eds.), Apoptosis: From Signaling Pathways to Therapeutic Tools. New York Academy of Sciences, New York, pp. 374-380.

Jacobs, M.N., Nolan, G.T., Hood, S.R., 2005. Lignans, bacteriocides and organochlorine compounds activate the human pregnane X receptor (PXR). Toxicol. Appl. Pharmacol. 209, 123-133.

Janer, G., Porte, C., 2007. Sex steroids and potential mechanisms of non-genomic endocrine disruption in invertebrates. Ecotoxicology 16, 145-160.

Jenab, M., Thompson, L.U., 1996. The influence of flaxseed and lignans on colon carcinogenesis and beta-glucuronidase activity. Carcinogenesis 17, 1343-1348.

Jiao, Y., Davin, L.B., Lewis, N.G., 1998. Furanofuran lignan metabolism as a function of seed maturation in Sesamum indicum: Methylenedioxy bridge formation. Phytochemistry 49, 387-394.

Jin, J.S., Kakiuchi, J., Hattori, M., 2007a. Enantioselective oxidation of enterodiol to enterolactone by human intestinal bacteria. Biol. Pharm. Bull. 30, 2204-2206.

Jin, J.S., Zhao, Y.F., Nakamura, N., Akao, T., Kakiuchi, N., Min, B.S., Hattori, M., 2007b. Enantioselective dehydroxylation of enterodiol and enterolactone precursors by human intestinal bacteria. Biol. Pharm. Bull. 30, 2113-2119.

Johnsson, P., Peerlkamp, N., Kamal-Eldin, A., Andersson, R.E., Andersson, R., Lundgren, L.N., Aman, P., 2002. Polymeric fractions containing phenol glucosides in flaxseed. Food Chem. 76, 207-212.

Jordan, V.C., 1995. Antiestrogens. In: Munson, P.L., Mueller, R.A., Breese, G.R. (Eds.), Principles of pharmacology:basic concepts \& clinical applications. Chapman \& Hall, New York, pp. 827-836.

Jungestrom, M.B., Thompson, L.U., Dabrosin, C., 2007. Flaxseed and its lignans inhibit estradiol-induced growth, angiogenesis, and secretion of vascular endothelial growth factor in human breast cancer xenografts in vivo. Clin. Cancer Res. 13, 1061-1067.

Kageyama, A., Benno, Y., Nakase, T., 1999. Phylogenetic evidence for the transfer of Eubacterium lentum to the genus Eggerthella as Eggerthella lenta gen. nov., comb. nov. Int. J. Syst. Bacteriol. 49, 1725-1732.

Kamal-Eldin, A., Appelqvist, L.Å. 1994. Variations in the composition of sterols, tocopherols and lignans in seed oils from four Sesamum species. J. Am. Oil Chem. Soc. 71, 149-156.

Kamal-Eldin, A., Appelqvist, L.Å., Yousif, G., 1994. Lignan analysis in seed oils from four Sesamum species: comparison of different chromatographic methods. J. Am. Oil. Chem. Soc. 71, 141-147.

Kamal-Eldin, A., Peerlkamp, N., Johnsson, P., Andersson, R., Andersson, R.E., Lundgren, L.N., Aman, P., 2001. An oligomer from flaxseed composed of secoisolariciresinoldiglucoside and 3-hydroxy-3-methyl glutaric acid residues. Phytochemistry 58, 587-590.

Kang, M.H., Naito, M., Tsujihara, N., Osawa, T., 1998. Sesamolin inhibits lipid peroxidation in rat liver and kidney. J. Nutr. 128, 1018-1022.

Katsuzaki, H., Osawa, T., Kawakishi, S., 1994. Chemistry and antioxidative activity of lignan glucosides in sesame seed. In: Ho, C.T., Osawa, T., Huang, M.T., Rosen, R.T. (Eds.), Food phytochemicals for cancer prevention II: Teas, spices and herbs. American chemical society, Washington, pp. 275-280.

Kiberstis, P., Smith, O., Norman, C., 2000. Bone health in the balance. Science 289, 1497.
Kiso, Y., 2004. Antioxidative roles of sesamin, a functional lignan in sesame seed, and it's effect on lipid- and alcohol-metabolism in the liver: A DNA microarray study. Biofactors 21, 191-196.

Kiso, Y., Tsuruoka, N., Kidokoro, A., Matsumoto, I., Abe, K., 2005. Sesamin ingestion regulates the transcription levels of hepatic metabolizing enzymes for alcohol and lipids in rats. Alcoholism (NY) 29, 116S-120S.

Kitts, D.D., Yuan, Y.V., Wijewickreme, A.N., Thompson, L.U., 1999. Antioxidant activity of the flaxseed lignan secoisolariciresinol diglycoside and its mammalian lignan metabolites enterodiol and enterolactone. Mol. Cell. Biochem. 202, 91-100.

Klinge, C.M., 2001. Estrogen receptor interaction with estrogen response elements. Nucleic Acids Res. 29, 2905-2919.

Klosterman, H.J., Smith, F., 1954. The isolation of beta-hydroxy-beta-methylglutaric acid from the seed of flax (Linum-usitatissimum). J. Am. Chem. Soc. 76, 1229-1230.

Klosterman, H.J., Smith, F., Clagett, C.O., 1955. The constitution of linocinnamarin. J. Am. Chem. Soc. 77, 420-421.

Korach, K.S., Migliaccio, S., Davis, V.L., 1995. Estrogens. In: Munson, P.L., Mueller, R.A., Breese, G.R. (Eds.), Principles of pharmacology: basic concepts \& clinical applications. Chapman \& Hall, New York, pp. 809-825.

Kranen, v.H.J., Mortensen, A., Sorensen, I.K., Berg-Wijnands van den, J., Beems, R., Nurmi, T., Adlercreutz, H., Kreijl, v.C.F., 2003. Lignan precursors from flaxseed or rye bran do not protect against the development if intestinal neoplasia in Apcmin mice. Nutr. Canc. Int. J. 45, 203-210.

Kuiper, G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., Gustafsson, J.A., 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 138, 863-870.

Lacey, M., Bohday, J., Fonseka, S.M.R., Ullah, A.I., Whitehead, S.A., 2005. Dose-response effects of phytoestrogens on the activity and expression of 3 beta-hydroxysteroid dehydrogenase and aromatase in human granulosa-luteal cells. J. Steroid Biochem. Mol. Biol. 96, 279-286.

Lajoie, S.F., Bank, S., Miller, T.L., Wolin, M.J., 1988. Acetate Production from Hydrogen and C-13 Carbon-Dioxide by the Microflora of Human Feces. Appl. Environ. Microbiol. 54, 2723-2727.

Lambert, N., Kroon, P.A., Faulds, C.B., Plumb, G.W., McLauchlan, W.R., Day, A.J., Williamson, G., 1999. Purification of cytosolic beta-glucosidase from pig liver and its reactivity towards flavonoid glycosides. Biochim. Biophys. Acta 110-116.

Lee, C.C., Chen, P.R., Lin, S., Tsai, S.C., Wang, B.W., Chen, W.W., Tsai, C.E., Shyu, K.G., 2004. Sesamin induces nitric oxide and decreases endothelin-1 production in HUVECs: possible implications for its anti hypertensive effect. J. Hypertens. 22, 23292338.

Lee, J.Y., Choe, E., 2006. Extraction of lignan compounds from roasted sesame oil and their effects on the autoxidation of methyl linoleatee. J. Food Sci. 71, C430-C436.

Lewis, J.E., Nickell, L.A., Thompson, L.U., Szalai, J.P., Kiss, A., Hilditch, J.R., 2006. A randomized controlled trial of the effect of dietary soy and flaxseed muffins on quality of life and hot flashes during menopause. Menopause-J. N. Am. Menopause Soc. 13, 631-642.

Liggins, J., Grimwood, R., Bingham, S.A., 2000. Extraction and quantification of lignan phytoestrogens in food and human samples. Anal. Biochem. 287, 102-109.

Liu, Z., Saarinen, N.M., Thompson, L.U., 2006. Sesamin is one of the major precursors of mammalian lignans in sesame seed (Sesamum indicum) as observed in vitro and in rats. J. Nutr. 136, 906-912.

Lonard, D.M., Smith, C.L., 2002. Molecular perspectives on selective estrogen receptor modulators (SERMs): progress in understanding their tissue- specific agonist and antagonist actions. Steroids 67, 15-24.

Lucas, E.A., Lightfoot, S.A., Hammond, L.J., Devareddy, L., Khalil, D.A., Daggy, B.P., Smith, B.J., Westcott, N., Mocanu, V., Soung, D.Y., Arjmandj, B.H., 2004. Flaxseed reduces plasma cholesterol and atherosclerotic lesion formation in ovariectomized Golden Syrian hamsters. Artherosclerosis 173, 223-229.

Madhusudhan, B., Wiesenborn, D., Schwarz, J., Tostenson, K., Gillespie, J., 2000. A dry mechanical method for concentrating the lignan secoisolariciresinol diglucoside in flaxseed. Food. Sci. Technol. 33, 268-275.

Mäkelä, T.H., Wähälä, K.T., Hase, T.A., 2000. Synthesis of enterolactone and enterodiol precursors as potential inhibitors of human estrogen synthetase (aromatase). Steroids 65, 437-441.

Marchand, P.A., Kato, M.J., Lewis, N.G., 1997. (+)-episesaminone, a Sesamum indicum furofuran lignan. Isolation and hemisynthesis. J. Nat. Prod. 60, 1189-1192.

Mazur, W., Fotsis, T., Wähälä, K., Ojala, S., Salakka, A., Adlercreutz, H., 1996. Isotope dilution gas chromatographic mass spectrometric method for the determination of isoflavonoids, coumestrol, and lignans in food samples. Anal. Biochem. 233, 169-180.

Mazur, W.M., Duke, J.A., Wähälä, K., Rasku, S., Adlercreutz, H., 1998. Isoflavonoids and lignans in legumes: nutritional and health aspects in humans. 9, 193-200.

Meagher, L.P., Beecher, G.R., Flanagan, V.P., Li, B.W., 1999. Isolation and characterization of the lignans, isolariciresinol and pinoresinol, in flaxseed meal. J. Agric. Food Chem. 47, 3173-3180.

Meagher, L.P., Beecher, R.G., 2000. Assessment of Data on the Lignan Content of Foods. 13, 935-947.

Milder, I.E.J., Arts, I.C.W., van de Putte, B., Venema, D.P., Hollman, P.C.H., 2005a. Lignan contents of Dutch plant foods: a database including lariciresinol, pinoresinol, secoisolariciresinol and matairesinol. Br. J. Nutr. 93, 393-402.

Milder, I.E.J., Feskens, E.J.M., Arts, I.C.W., de Mesquita, H.B.B., Hollman, P.C.H., Kromhout, D., 2005b. Intake of the plant lignans secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol in Dutch men and women. J. Nutr. 135, 1202-1207.

Min, T.P., Kasahara, H., Bedgar, D.L., Youn, B.Y., Lawrence, P.K., Gang, D.R., Halls, S.C., Park, H.J., Hilsenbeck, J.L., Davin, L.B., Lewis, N.G., Kang, C., 2003. Crystal structures of pinoresinol-lariciresinol and phenylcoumaran benzylic ether reductases and their relationship to isoflavone reductases. J. Biol. Chem. 278, 50714-50723.

Miyake, Y., Fukumoto, S., Okada, M., Sakaida, K., Nakamura, Y., Osawa, T., 2005. Antioxidative catechol lignans converted from sesamin and sesaminol triglucoside by culturing with Aspergillus. J. Agric. Food Chem. 53, 22-27.

Moazzami, A.A., Andersson, R.E., Kamal-Eldin, A., 2006a. Characterization and analysis of sesamolinol diglucoside in sesame seeds. Biosci. Biotechnol. Biochem. 70, 1478-1481.

Moazzami, A.A., Andersson, R.E., Kamal-Eldin, A., 2006b. HPLC analysis of sesaminol glucosides in sesame seeds. J. Agric. Food Chem. 54, 633-638.

Moazzami, A.A., Kamal-Eldin, A., 2006. Sesame seed is a rich source of dietary lignans. J. Am. Oil Chem. Soc. 83, 719-723.

Moinuddin, S.G.A., Youn, B., Bedgar, D.L., Costa, M.A., Helms, G.L., Kang, C., Davin, L.B., Lewis, N.G., 2006. Secoisolariciresinol dehydrogenase: mode of catalysis and stereospecificity of hydride transfer in Podophyllum peltatum. Org. Biomol. Chem. 4, 808816.

Moss, G.P., 2000. Nomenclature of lignans and neolignans (IUPAC Recommendations 2000). Pure Appl. Chem. 72, 1493-1523.

Mosselman, S., Polman, J., Dijkema, R., 1996. ER beta: Identification and characterization of a novel human estrogen receptor. FEBS Lett. 392, 49-53.

Mueller, S.O., Simon, S., Chae, K., Metzler, M., Korach, K.S., 2004. Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor alpha (ER alpha) and ER beta in human cells (vol 80, pg 14, 2004). Toxicol. Sci. 81, 530-531.

Muller, V., Imkamp, F., Rauwolf, A., Kusel, K., Drake, H.L., 2004. Molecular and cellular biology of acetogenic bacteria. In: Nakano, M.M., Zuber, P. (Eds.), Strict and facultative anaerobes: Medical and environmental aspects. Horizon Bioscience, Wymondham, pp. 251281.

Muramatsu, M., Inoue, S., 2000. Estrogen receptors: How do they control reproductive and nonreproductive functions? Biochem. Biophys. Res. Comm. 270, 1-10.

Nakai, M., Harada, M., Nakahara, K., Akimoto, K., Shibata, H., Miki, W., Kiso, Y., 2003. Novel antioxidative metabolites in rat liver with ingested sesamin. J. Agric. Food Chem. 51, 1666-1670.

Nakano, D., Kwak, C.J., Fujii, K., Ikemura, K., Satake, A., Ohkita, M., Takaoka, M., Ono, Y., Nakai, M., Tomimori, N., Kiso, Y., Matsumura, Y., 2006. Sesamin metabolites induce an endothelial nitric oxide-dependent vasorelaxation through their antioxidative propertyindependent mechanisms: Possible involvement of the metabolites in the antihypertensive effect of sesamin. J. Pharmacol. Exp. Ther. 318, 328-335.

Nakatsubo, T., Mizutani, M., Suzuki, S., Hattori, T., Umezawa, T., 2008. Characterization of Arabidopsis thaliana pinoresinol reductase, a new type of enzyme involved in lignan biosynthesis. J. Biol. Chem. 283, 15550-15557.

Namiki, M., 1995. The Chemistry and Physiological Functions of Sesame. Food Rev. Int. 11, 281-329.

Namiki, M., 2007. Nutraceutical functions of sesame: A review. Crit. Rev. Food Sci. Nutr. 47, 651-673.

Nemeth, K., Plumb, G.W., Berrin, J.G., Juge, N., Jacob, R., Naim, H.Y., Williamson, G., Swallow, D.M., Kroon, P.A., 2003. Deglycosylation by small intestinal epithelial cell betaglucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans. Eur. J. Nutr. 42, 29-42.

Niemeyer, H.B., Metzler, M., 2003. Differences in the antioxidant activity of plant and mammalian lignans. J. Food Eng. 56, 255-256.

Noguchi, A., Fukui, Y., Iuchi-Okada, A., Kakutani, S., Satake, H., Iwashita, T., Nakao, M., Umezawa, T., Ono, E., 2008. Sequential glucosylation of a furofuran lignan, (+)-sesarninol, by Sesamum indicum UGT71A9 and UGT94D1 glucosyltransferases. Plant J. 54, 415-427.

Oikarinen, S., Heinonen, S.M., Nurmi, T., Adlercreutz, H., Mutanen, M., 2005. No effect on adenoma formation in Min mice after moderate amount of flaxseed. Eur. J. Nutr. 44, 273-280.

Ono, E., Nakai, M., Fukui, Y., Tomimori, N., Fukuchi-Mizutani, M., Saito, M., Satake, H., Tanaka, T., Katsuta, M., Umezawa, T., Tanaka, Y., 2006. Formation of two methylenedioxy bridges by a Sesamum CYP81Q protein yielding a furofuran lignan, (+)sesamin. Proc. Natl. Acad. Sci. U. S. A. 103, 10116-10121.

Osawa, T., Nagata, M., Namiki, M., Fukuda, Y., 1985. Sesamolinol, a novel antioxidant isolated from sesame seeds. 49, 3351-3352.

Pan, A., Sun, J.Q., Chen, Y.Q., Ye, X.W., Li, H.X., Yu, Z.J., Wang, Y.F., Gu, W.J., Zhang, X.Y., Chen, X.F., Demark-Wahnefried, W., Liu, Y., Lin, X., 2007. Effects of a flaxseedderived lignan supplement in type 2 diabetic patients: A randomized, double-blind, crossover trial. PLos Clin. Trials 2, 1-7.

Penalvo, J.L., Heinonen, S.M., Aura, A.M., Adlercreutz, H., 2005. Dietary sesamin is converted to enterolactone in humans. J. Nutr. 135, 1056-1062.

Penttinen, P., Jaehrling, J., Damdimopoulos, A.E., Inzunza, J., Lemmen, J.G., van der Saag, P., Pettersson, K., Gauglitz, G., Makela, S., Pongratz, I., 2007. Diet-derived polyphenol metabolite enterolactone is a tissue-specific estrogen receptor activator. Endocrinology 148, 4875-4886.

Penumathsa, S.V., Koneru, S., Thirunavukkarasu, M., Zhan, L.J., Prasad, K., Maulik, N., 2007. Secoisolariciresinol diglucoside: Relevance to angiogenesis and cardioprotection against ischemia-reperfusion injury. J. Pharmacol. Exp. Ther. 320, 951-959.

Picherit, C., Coxam, V., Bennetau-Pelissero, C., Kati-Coulibaly, S., Davicco, M.-J., Lebecque, P., Barlet, J.-P., 2000. Daidzein is more efficient than genistein in preventing ovariectomy-induced bone loss in rats. J. Nutr. 130, 1675-1681.

Pike, A.C.W., Brzozowski, A.M., Hubbard, R.E., Bonn, T., Thorsell, A.G., Engström, O., Ljunggren, J., Gustafsson, J.Å., Carlquist, M., 1999. Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. EMBO J. 18, 4608-4618.

Power, K.A., Ward, W.E., Chen, J.M., Saarinen, N.M., Thompson, L.U., 2006. Genistein alone and in combination with the mammalian lignans enterolactone and enterodiol induce estrogenic effects on bone and uterus in a postmenopausal breast cancer mouse model. Bone 39, 117-124.

Prasad, K., 1997. Dietary flax seed in prevention of hypercholesterolemic atherosclerosis. Atherosclerosis 132, 69-76.

Prasad, K., 2000. Antioxidant activity of secoisolariciresinol diglucoside-derived metabolites, secoisolariciresinol, enterodiol and enterolactone. Int. J. Angiol. 9, 220-225.

Qiu, S.-X., Lu, Z.-Z., Luyengi, L., Lee, S.K., Pezzuto, J.M., Farnsworth, N.R., Thompson, L.U., Fong, H.H.S., 1999. Isolation and characterization of flaxseed (Linum usitatissimum) constituents. Pharm. Biol. 37, 1-7.

Schöttner, M., Gansser, D., Spiteller, G., 1997. Lignans from the roots of Urtica dioica and their metabolites bind to human sex hormone binding globulin (SHBG). Planta Med. 63, 529-532.

Serraino, M., Thompson, L.U., 1992. Flaxseed Supplementation and Early Markers of Colon Carcinogenesis. Cancer Lett. 63, 159-165.

Setchell, K.D.R., Lawson, A.M., Mitchell, F.L., Adlercreutz, H., Kirk, D.N., Axelson, M., 1980. Lignans in man and in animal species. Nature 287, 740-742.

Setchell, K.D.R., Lawson, A.M., Borriello, S.P., Harkness, R., Gordon, H., Morgan, D.M.L., Kirk, D.N., Adlercreatz, H., Anderson, L.C., Axelson, M., 1981. Lignan formation in man-microbial involvement and possible roles in relation to cancer. Lancet 2, 4-7.

Shyu, Y.S., Hwang, L.S., 2002. Antioxidative activity of the crude extract of lignan glycosides from unroasted Burma black sesame meal. Food Res. Int. 35, 357-365.

Sicilia, T., Niemeyer, H.B., Honig, D.M., Metzler, M., 2003. Identification and stereochemical characterization of lignans in flaxseed and pumpkin seeds. J. Agric. Food Chem. 51, 1181-1188.

Smeds, A.I., Eklund, P.C., Sjoholm, R.E., Willfor, S.M., Nishibe, S., Deyama, T., Holmbom, B.R., 2007. Quantification of a broad spectrum of lignans in cereals, oilseeds, and nuts. J. Agric. Food Chem. 55, 1337-1346.

Stitch, S.R., Toumba, J.K., Groen, M.B., Funke, C.W., Leemhuis, J., Vink, J., Woods, G.F., 1980. Excretion, isolation and structure of a new phenolic constituent of female urine. Nature 287, 738-740.

Suja, K.P., Jayalekshmy, A., Arumughan, C., 2004. Free radical scavenging behavior of antioxidant compounds of sesame (Sesamum indicum L.) in DPPH center dot system. J. Agric. Food Chem. 52, 912-915.

Suja, K.P., Jayalekshmy, A., Arumughan, C., 2005. In vitro studies on antioxidant activity of lignans isolated from sesame cake extract. J. Sci. Food Agric. 85, 1779-1783.

Suzuki, S., Umezawa, T., 2007. Biosynthesis of lignans and norlignans. J. Wood Sci. 53, 273-284.

Thompson, L.U., Rickard, S.E., Orcheson, L.J., Seidl, M.M., 1996. Flaxseed and its lignan and oil components reduce mammary tumor growth at a late stage of carcinogenesis. Carcinogenesis 17, 1373-1376.

Thompson, L.U., Chen, J.M., Li, T., Strasser-Weippl, K., Goss, P.E., 2005. Dietary flaxseed alters tumor biological markers in postmenopausal breast cancer. Clin. Cancer Res. 11, 3828-3835.

Umeda-Sawada, R., Ogawa, M., Igarashi, O., 1999. The metabolism and distribution of sesame lignans (sesamin and episesamin) in rats. Lipids 34, 633-637.

Umezawa, T., Davin, L.B., Yamamoto, E., Kingston, D.G.I., Lewis, N.G., 1990. Lignan Biosynthesis in Forsythia Species. J. Chem. Soc. Chem. Commun. 1405-1408.

Umezawa, T., 2003. Phylogenetic distribution of lignan producing plants. Wood res. 90, 27110.
von Heimendahl, C.B.I., Schafer, K.M., Eklund, P., Sjoholm, R., Schmidt, T.J., Fuss, E., 2005. Pinoresinol-lariciresinol reductases with different stereospecificity from Linum album and Linum usitatissimum. Phytochemistry 66, 1254-1263.

Wang, C.F., Makela, T., Hase, T., Adlercreutz, H., Kurzer, M.S., 1994. Lignans and Flavonoids Inhibit Aromatase Enzyme in Human Preadipocytes. J. Steroid Biochem. Mol. Biol. 50, 205-212.

Wang, L., Chen, J.M., Thompson, L.U., 2005. The inhibitory effect of flaxseed on the growth and metastasis of estrogen receptor negative human breast cancer xenografts is attributed to both its lignan and oil components. Int. J. Cancer 116, 793-798.

Wang, L.-Q., Meselhy, M.R., Li, Y., Qin, G.-W., Hattori, M., 2000. Human intestinal bacteria capable of transforming secoisolariciresinol diglucoside to mammalian lignans, enterodiol and enterolactone. Chem. Pharm. Bull. 48, 1606-1610.

Ward, W.E., Yuan, Y.V., Cheung, A.M., Thompson, L.U., 2001. Exposure to purified lignan from flaxseed (Linum usitatissimum) alters bone development in female rats. Br. J. Nutr. 86, 499-505.

Waters, A.P., Knowler, J.T., 1982. Effect of a lignan (HPMF) on RNA synthesis in the rat uterus. J. Reprod. Fertility 66, 379-381.

Webb, A.L., McCullough, M.L., 2005. Dietary lignans: Potential role in cancer prevention. Nutr. Cancer 51, 117-131.

Wu, W.H., Kang, Y.P., Wang, N.H., Jou, H.J., Wang, T.A., 2006. Sesame ingestion affects sex hormones, antioxidant status, and blood lipids in postmenopausal women. J. Nutr. 136, 1270-1275.

Xia, Z.Q., Costa, M.A., Proctor, J., Davin, L.B., Lewis, N.G., 2000. Dirigent-mediated podophyllotoxin biosynthesis in Linum flavum and Podophyllum peltatum. Phytochemistry 55, 537-549.

Xia, Z.Q., Costa, M.A., Pelissier, H.C., Davin, L.B., Lewis, N.G., 2001.
Secoisolariciresinol dehydrogenase purification, cloning, and functional expression Implications for human health protection. J. Biol. Chem. 276, 12614-12623.

Xu, X., Wang, H.-J., Murphy, P.A., Cook, L., Hendrich, S., 1994. Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. J. Nutr. 124, 825-832.

Yamauchi, S., Ina, T., Kirikihira, T., Masuda, T., 2004. Synthesis and antioxidant activity of oxygenated furofuran lignans. 68, 183-192.

Yamauchi, S., Sugahara, T., Nakashima, Y., Okada, A., Akiyama, K., Kishida, T., Maruyama, M., Masuda, T., 2006. Radical and superoxide scavenging activities of matairesinol and oxidized matairesinol. Biosci. Biotechnol. Biochem. 70, 1934-1940.

Youn, B.Y., Moinuddin, S.G.A., Davin, L.B., Lewis, N.G., Kang, C.H., 2005. Crystal structures of apo-form and binary/ternary complexes of Podophyllum secoisolariciresinol dehydrogenase, an enzyme involved in formation of health-protecting and plant defense lignans. J. Biol. Chem. 280, 12917-12926.

Zhang, W., Wang, X., Liu, Y., Tian, H., Flickinger, B., Empie, M.W., Sung, S.Z., 2008. Dietary flaxseed lignan extract lowers plasma cholesterol and glucose concentrations in hypercholesterolaemic subjects. Br. J. Nutr. 99, 1301-1309.

Zoetendal, E.G., Vaughan, E.E., de Vos, W.M., 2006. A microbial world within us. Mol. Microbiol. 59, 1639-1650.

## Chapter 2 <br> The flavonoid herbacetin diglucoside as a constituent of the lignan macromolecule from flaxseed hulls



Chapter 2


#### Abstract

Lignans in flaxseed are known to be part of a macromolecule in which they are connected through the linker-molecule hydroxyl-methyl-glutaric acid (HMGA). In this study, the lignan macromolecule was extracted from flaxseed hulls and degraded to its monomeric constituents by complete saponification. Besides secoisolariciresinol diglucoside (SDG), the phenolic compounds $p$-coumaric acid glucoside (CouAG) and ferulic acid glucoside (FeAG) were isolated, which was expected based on indications from literature. Also the flavonoid herbacetin diglucoside (HDG) was found. The presence of HDG was confirmed by NMR following preparative RP-HPLC purification. Also the presence of the three other constituents (CouAG, FeAG and SDG) was confirmed by NMR. To prove that HDG is a substructure of the lignan macromolecule, the macromolecule was fragmented by partial saponification. A fragment consisting of HDG and HMGA was indicated. This fragment was isolated by preparative RP-HPLC and its identity was confirmed by NMR. It is concluded that the flavonoid HDG is a substructure of the lignan macromolecule from flaxseed hulls and that it is incorporated in the macromolecule via the same linker-molecule as SDG.


## Introduction

Flaxseed is increasingly used as an ingredient in food products (Oomah, 2001), because of its high $\alpha$-linolenic acid and dietary fiber content (Bhatty and Cherdkiatgumchai, 1990; Bhatty, 1995), but recently also because of its secondary metabolites. Important secondary metabolites are lignans, which are present in flaxseed in a higher concentration than in other edible sources (Liggins et al., 2000; Mazur and Adlercreutz, 1998; Milder et al., 2005). Lignans are reported to exhibit protective effects against hormone-related types of cancer like breast cancer (Boccardo et al., 2004; Chen et al., 2002; Chen and Thompson, 2003; Thompson et al., 1996a,b) and against non-hormone related colon cancer (Sung et al., 1998). Furthermore, they lower the risk of cardiovascular diseases (Lucas et al., 2004; Vanharanta et al., 1999).

The main lignan in flaxseed is secoisolariciresinol diglucoside (SDG), which is present in defatted flaxseed flour in concentrations up to $3 \%(\mathrm{w} / \mathrm{w}$ ) (Bakke and Klosterman, 1956; Johnsson et al., 2000). Other lignans present in flaxseed are matairesinol (MAT) (Liggins et al., 2000), isolariciresinol (isoLARI) (Meagher et al., 1999), pinoresinol (PINO) (Meagher et al., 1999), and lariciresinol (LARI) (Sicilia et al., 2003). Other phenolic compounds reported in flaxseed, which might contribute to the health effects ascribed to flaxseeds, are hydroxycinnamic acids like p-coumaric acid (Klosterman et al., 1955), ferulic acid, sinapic acid, caffeic acid (Dabrowski and Sosulski, 1984) and their glucosides, and the flavonoids herbacetin diglucoside (HDG) and kaempferol diglucoside (KDG) (Qiu et al., 1999). The structures of the most relevant compounds are shown in Fig. 1A.

The lignans in flaxseed are part of an oligomeric structure (Kamal-Eldin et al., 2001), which is referred to as the lignan macromolecule. It is reported (Kamal-Eldin et al., 2001; Klosterman and Smith, 1954) that this lignan macromolecule consists of SDG units that are ester-linked by hydroxy-methyl-glutaric acid (HMGA) (see Fig. 1B). The molecular weight of this lignan macromolecule was estimated to be around 4000 based on the intensity of NMR signals (Kamal-Eldin et al., 2001), but further experimental evidence is lacking. In addition to SDG, $p$-coumaric acid glucoside (CouAG) and ferulic acid glucoside (FeAG) were reported to be released from the lignan macromolecule after alkali treatment (Johnsson et al., 2002; Kamal-Eldin et al., 2001). However, these components were neither included (Kamal-Eldin et al., 2001) nor annotated (Johnsson et al., 2002) as integral constituents of the lignan macromolecule. For the other compounds identified in flaxseed, there are no indications that they are part of the lignan macromolecule. The hulls, which comprise about $40 \%$ of the seed, are enriched in SDG compared to the cotyledons (Madhusudhan et al., 2000; Oomah and Mazza, 1997; Wiesenborn et al., 2003). However, no information is available concerning the presence of a lignan macromolecule in flaxseed hulls. In this study, it is shown that the lignan macromolecule is present in flaxseed hulls and that it contains flavonoidic constituents besides the already known constituents SDG, CouAG en FeAG.

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(A)
(1)

(2)


(3)

(4)

(B)


Figure 1. (A) Structures of the main phenolic constituents of flaxseed: $1=$ CouAG, $2=$ FeAG, $3=$ HDG, 4 = SDG, 5 = HMGA. All glucosyl-moieties are coupled via their C-6. Annotations will be used throughout the text. (B) Structure of the lignan macromolecule from flaxseed: $\mathrm{R}_{1}=H$ or SDG, $\mathrm{R}_{2}=\mathrm{OH}$ or HMGA.

## Results

## Identification of the constituents of the lignan macromolecule

Flaxseed hulls were used as a source of lignans because of their high lignan content (Oomah and Mazza, 1997). Hulls were extracted with aq. EtOH aiming at the extraction of a lignan macromolecule. From 400 g flaxseed hulls, 29.9 g of extract was obtained, accounting for $7.5 \%(\mathrm{w} / \mathrm{w})$ of the dry weight of the hulls. To obtain information about the composition of this extract, it was degraded by alkali treatment. Under alkaline conditions the ester-linkages are degraded while the glycosidic bonds are stable. In this way SDG and other glycosides are released, providing information on the native constituents of the lignan macromolecule.
Analysis of the untreated extract ( 0 mM NaOH ) on GPC (Fig. 2) and RP-HPLC (Fig. 3) showed that the extract eluted as one broad peak indicating a heterogenic macromolecular structure. Saponification with increasing concentrations of NaOH resulted in the progressive degradation of this macromolecule as shown by the GPC (Fig. 2) and the RPHPLC/MS results (Fig. 3). With increasing concentrations of NaOH ( $1-10 \mathrm{mM}$ ), the retention time of the saponified macromolecule on GPC (Fig. 2) was increased as compared to the retention time of the intact macromolecule, pointing at a decrease in fragment size upon saponification. Except for a small increase of the peak eluting around 29 min , the chromatogram of the macromolecule saponified with 75 mM NaOH was similar to the chromatogram of the macromolecule saponified with 25 mM NaOH . Therefore, it was assumed that with 75 mM NaOH the macromolecule was degraded completely or could not be degraded further.


Figure 2. GPC elution profile of lignan macromolecule saponified with a range of NaOH concentrations.

Also on RP-HPLC (Fig. 3), the sample saponified with 75 mM NaOH showed a similar fragmentation pattern compared to the pattern of sample saponified with 25 mM NaOH , apart from some variation in the ratio between peak 1 and 2, which was also observed in Fig. 4 (see further). The MS analysis of the compounds separated on RP-HPLC showed that upon saponification with 75 mM NaOH the monomeric constituents were liberated. Peak 1 was annotated as CouAG ( $\mathrm{m} / \mathrm{z}=325.0[\mathrm{M}-\mathrm{H}]^{-}$), peak 2 as FeAG $\left(\mathrm{m} / \mathrm{z}=354.9[\mathrm{M}-\mathrm{H}]^{-}\right)$, peak 3 as HDG ( $\mathrm{m} / \mathrm{z}=625.2[\mathrm{M}-\mathrm{H}]^{-}$), and peak 4 as SDG ( $\mathrm{m} / \mathrm{z}=685.3[\mathrm{M}-\mathrm{H}]^{-}$).
The heterogeneity of the lignan macromolecule was confirmed by MALDI-TOF MS analysis of the unsaponified extract. A complex spectrum (not shown) was obtained showing clusters of peaks in which molecules ranging from 2SDG+1HMGA ( $\mathrm{m} / \mathrm{z}=1521.8$ $\left.[\mathrm{M}+\mathrm{Na}]^{+}\right)$to $5 \mathrm{SDG}+5 \mathrm{HMGA}\left(\mathrm{m} / \mathrm{z}=4104.8[\mathrm{M}+\mathrm{Na}]^{+}\right)$could be annotated.
These results showed that the aq. EtOH extract consisted of a heterogenic macromolecule with SDG as one of its constituents, showing that a lignan macromolecule was present in flaxseed hulls. Next, the annotation of the flavonoid HDG is noteworthy, since HDG was isolated from flaxseeds only once before (Qiu et al., 1999).


Figure 3. Analytical RP-HPLC elution profiles of lignan macromolecule saponified with a range of NaOH concentrations: $1=$ CouAG; $2=$ FeAG; 3 = HDG; $4=\mathrm{SDG} ; \mathrm{LM}=$ lignan macromolecule.

To be able to confirm the identity of HDG and the other monomeric constituents by NMR, they were purified from the fully saponified lignan macromolecule. Therefore, the analytical RP-HPLC procedure was up-scaled. The preparative RP-HPLC elution profile (Fig. 4) showed high similarity with the one obtained by analytical chromatography (Fig. 3). Fractions of interest were collected as indicated in Fig. 4. The identity of the 4 fractions was confirmed based on their retention time on analytical RP-HPLC and MS analysis. Starting with 2.5 g fully saponified lignan macromolecule, 0.3 g CouAG (fraction $1, \mathrm{~m} / \mathrm{z}=$


Figure 4. Preparative RP-HPLC elution profiles of fully saponified lignan macromolecule ( 75 mM NaOH ). The collected fractions are indicated; fraction $1=$ CouAG, fraction $2=$ FeAG, fraction 3 = HDG, fraction $4=$ SDG. In the inserts, the analytical RP-HPLC elution profiles of the four fractions after semi-preparative purification are shown.
$325.0[\mathrm{M}-\mathrm{H}]^{-}$), 0.1 g FeAG (fraction $2, \mathrm{~m} / \mathrm{z}=354.9[\mathrm{M}-\mathrm{H}]^{-}$), 0.1 g HDG (fraction $3, \mathrm{~m} / \mathrm{z}=$ $625.2[\mathrm{M}-\mathrm{H}]^{-}$), and 0.9 g SDG (fraction $4, \mathrm{~m} / \mathrm{z}=685.3[\mathrm{M}-\mathrm{H}]$ ) was collected after preparative RP-HPLC.
The purity of the fractions was determined by analytical RP-HPLC. Based on the area percentage of the main peak, most fractions were not sufficiently pure for NMR analysis (purity ranging from 62-91\%). Therefore, a second purification step was performed on semi-preparative RP-HPLC. The compounds of interest and the impurities could be separated resulting in more pure fractions. This is shown in the inserts of Fig. 4 in which the analytical RP-HPLC profiles of the purified fractions collected by semi-preparative purification are presented. FeAG (fraction 2*) and SDG (fraction $4^{*}$ ) were $96 \%$ and $95 \%$ pure, respectively, based on the $\mathrm{UV}_{280 n \mathrm{~nm}}$ responses. In addition, the MS results showed solely $\mathrm{m} / \mathrm{z}$-ratios corresponding to FeAG and SDG. CouAG (fraction 1*) was $97 \%$ pure based on $\mathrm{UV}_{280 \mathrm{~nm}}$ responses but MS analysis showed that besides CouAG ( $\mathrm{m} / \mathrm{z}=324.8$ [M-$\mathrm{H}]^{-}$), a compound with an $\mathrm{m} / \mathrm{z}$-ratio of 340.9 ( $[\mathrm{M}-\mathrm{H}]^{-}$) was present. This compound was annotated as caffeic acid glucoside. The purity of the main peak of HDG (fraction 3*) based on $U V_{280 n m}$ response was $71 \%$. This is explained by the presence of two shoulders flanking the main peak of HDG. MS analysis of fraction 3* showed the presence of only HDG.

Despite the remaining impurities, the identity of these components could be confirmed by NMR. The chemical shifts observed for FeAG, CouAG, and SDG (see spectral data in experimental section) were as reported in literature (Johnsson et al., 2002; Kamal-Eldin et al., 2001). For HDG, both the ${ }^{1} \mathrm{H}$ and ${ }^{13} \mathrm{C}$ chemical shifts are shown in Table 1 (see Fig. 1

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for the annotations). The chemical shifts for the aglycon were similar as reported by Qiu and coworkers (1999). The positions of both $\beta$-glucopyranose moieties on the C-3 and C-8 of the aglycon were proven by the presence of a cross peak in the HMBC spectrum between $\mathrm{C}-3$ and $\mathrm{H}-1$ ", and $\mathrm{C}-8$ and $\mathrm{H}-1^{\prime \prime \prime}$. It has to be noted that there is a slight difference in chemical shifts compared to literature since the spectra were obtained in $\mathrm{CD}_{3} \mathrm{OD}$ instead of DMSO-d6 solution.
These results showed that besides SDG, CouAG and FeAG, HDG was released from the lignan macromolecule after alkali treatment. This suggests that also flavonoids are part of the macromolecular lignan structure.

## Identification of HDG as a part of the lignan macromolecule

To obtain further proof for HDG being part of the lignan macromolecule, an oligomeric fragment containing HDG was needed. Therefore, partially fragmented lignan macromolecule was subjected to preparative RP-HPLC. For this, lignan macromolecule partially saponified with 2 mM NaOH was selected, because it resulted in the broadest range of fragments (Fig. 3). In the samples treated with less than 2 mM NaOH , intact macromolecule was still present. In the samples saponified with 6 mM NaOH or more, the monomeric constituents became predominant and the structural diversity was less.
During preparative RP-HPLC of the partially saponified lignan macromolecule, fractions were collected as indicated in Fig. 5. Based on MS analysis, fraction 3 was annotated as monomeric HDG. In fraction 4, an $m / z$-ratio of 769.1 ([M-H] $]^{-}$) was found which could be annotated as HDG+HMGA. As this fraction was dominated by SDG it was not investigated further. In fraction 5, a fragment with an $\mathrm{m} / \mathrm{z}$-ratio of 797.2 ([M-H]) was annotated as HDG $+\mathrm{HMGA}+\mathrm{EtOH}$.
Fraction 5 was not pure and was subjected to semi-preparative purification. The result of the analytical analysis of the fraction, which included the HDG containing fragment, obtained after semi-preparative purification is shown in the insert in Fig. 5. This fraction (fraction $5^{*}$ ) was $92 \%$ pure based on the $\mathrm{UV}_{280 \mathrm{~nm}}$ response. MS analysis showed the presence of several $\mathrm{m} / \mathrm{z}$-ratios (data not shown), one of them corresponding to the fragment of HDG+HMGA+EtOH $\left(\mathrm{m} / \mathrm{z}=797.1[\mathrm{M}-\mathrm{H}]^{-}\right)$. No further efforts were made to further purify this fraction.
The NMR-data of the fragment of HDG+HMGA+EtOH are shown in Table 1 (see Fig. 1 for the annotations). The presence of HDG in the fragment was proven by obtaining the same chemical shifts for the aglycon as in monomeric HDG. Again, the $\beta$-glucopyranose was linked to both the C-3 and the C-8 of the aglycon. HMGA was recognized by the peaks between 2.6 and $2.3 \mathrm{ppm}(\beta$ and $\delta$ ) and the presence of cross peaks in the HMBC spectrum between these peaks and $\mathrm{C}-\alpha$ and $\varepsilon$ at 172 ppm. These two downfield shifted carbons are typical for an esterified carboxylic acid group.
The HMGA molecule is linked to the glucose at C-6 via an ester linkage between C- $\alpha$ and C-6', proven by the presence of 2 cross peaks in the HMBC spectrum between C- $\alpha$ and H6a'' and b’', and by the significantly downfield shifted proton and carbon chemical shifts of H6a'' and b', HMGA is esterified with EtOH via C- - as shown by the presence of a cross-peak in the HMBC spectrum between C- $\varepsilon$ and the $\mathrm{CH}_{2}$ belonging to ethanol. To obtain further information about the linkage of HDG via HMGA to the lignan macromolecule, the samples obtained by partial saponification were screened for $\mathrm{m} / \mathrm{z}$-ratios
corresponding to HDG+2HMGA ( $\mathrm{m} / \mathrm{z}=913.2[\mathrm{M}-\mathrm{H}]^{-}$) and for HDG+HMGA+SDG ( $\mathrm{m} / \mathrm{z}=$ $1437.3[\mathrm{M}-\mathrm{H}]^{-}$).

Table 1. NMR data of herbacetin-di- $\beta$-glucopyranoside and herbacetin-di- $\beta$-gluco-pyranoside + hydroxy-methyl-glutaric acid + ethanol. In Fig. 1 the annotations are given.

| Position | HDG |  | HDG+HMGA+EtOH |  |
| :---: | :---: | :---: | :---: | :---: |
|  | ${ }^{1} \mathrm{H}$ | ${ }^{13} \mathrm{C}$ | ${ }^{1} \mathrm{H}$ | ${ }^{13} \mathrm{C}$ |
|  | $\delta$, mult., $J(\mathrm{~Hz})$ | $\delta$ | $\delta$, mult., $J(\mathrm{~Hz})$ | $\delta$ |
| 2 | - | 159.0 | - | 159.0 |
| 3 | - | 135.3 | - | 135.4 |
| 4 | - | nd | - | nd |
| 5 | - | 158.8 | - | 158.9 |
| 6 | 6.28, s | 100.1 | 6.30,s | 100.3 |
| 7 | - | 158.8 | - | 158.9 |
| 8 | - | 126.4 | - | 126.6 |
| 9 | - | nd | - | nd |
| 10 | - | 105.8 | - | 105.7 |
| 1 ' | - | 122.7 | - | 122.5 |
| 2 ' | 8.37, d, 8.8 | 133.1 | 8.39, d, 8.9 | 133.1 |
| 3 ' | 6.92, d, 8.8 | 116.0 | 6.92, d, 8.9 | 116.2 |
| 4' |  | 161.6 | - | 161.8 |
| 5' | 6.92, d, 8.8 | 116.0 | 6.92, d, 8.9 | 116.2 |
| 6 ' | 8.37, d, 8.8 | 133.1 | 8.39, d, 8.9 | 133.1 |
| 1', | 5.32, d, 7.6 | 103.8 | 5.30, d, 7.7 | 103.9 |
| 2', | 3.48 | 75.8 | nd | 75.5 |
| 3', | 3.41 | 77.9 | nd | 77.7 |
| 4', | 3.33 | 71.6 | nd | 71.0 |
| 5', | nd | 78.6 | nd | 78.3 |
| 6a'' | 3.64 | 62.7 | 4.27 | 64.7 |
| 6b"' | 3.77 |  | 4.12 |  |
| 1,', | 4.74, d, 7.9 | 107.7 | 4.75, $d, 7.8$ | 107.8 |
| 2',' | 3.58 | 75.3 | nd | 75.9 |
| 3', | 3.43 | 77.9 | nd | 77.9 |
| 4,', | 3.53 | 70.8 | nd | 71.2 |
| 5,', | 3.42 | 78.6 | nd | 78.1 |
| 6a'" | 3.74 | 62.3 | 3.74 | 62.3 |
| 6b"' | 3.82 |  | 3.83 |  |
| $\alpha$ |  |  | - | 172.0 |
| $\beta$ a |  |  | 2.56 | 45.9 |
| $\beta$ b |  |  | 2.42 |  |
| $\gamma$ |  |  | - | 70.7 |
| סa |  |  | 2.47 | 45.9 |
| ¢b |  |  | 2.38 |  |
| $\varepsilon$ |  |  | - | 172.7 |
| $\omega \mathrm{a}$ |  |  | 1.12, s | 27.9 |
| $\mathrm{CH}_{2}$ |  |  | 4.02 | 61.6 |
| $\mathrm{CH}_{3}$ |  |  | 1.17, $t, 7.2$ | 14.6 |

No $m / z$-ratio of $913.2+/-0.5$ ([M-H] $)$ was found, but in fraction 6 (Fig. 5) the $m / z$-ratio of 1437.3 ([M-H] ${ }^{-}$) was present. Fraction 6 was subjected to MS/MS fragmentation. Fragments with $\mathrm{m} / \mathrm{z}$-ratios of 463.1, 625.2, and 1275.3 [M-H] ${ }^{-}$, annotated as herbacetin monoglucoside, HDG, and HDG+HMGA+SDG minus a glucosyl residue, respectively, were identified. These results are in accordance with the annotation of this $m / z$-ratio as HDG+HMGA+SDG. Since this fragment was present in low quantities, it was not purified. In the peaks eluting between 12 and 15 min . (Fig. 5), no indications of fragments containing HDG were found, and therefore these fragments were not investigated further.


Figure 5. Preparative RP-HPLC elution profiles of partially saponified lignan macromolecule ( 2 mM $\mathrm{NaOH})$. The collected fractions are indicated; fraction $3=$ HDG, fraction $4=$ SDG, fraction $5=$ HDG+HMGA+EtOH, fraction $6=$ SDG+2HMGA. In the insert, the analytical RP-HPLC profile of the HDG containing fragment (fraction 5) after semi-preparative purification is shown.

## Discussion

This study identified the flavonol HDG as one of the constituents of the lignan macromolecule in flax seed hulls. Qiu and coworkers (1999) already reported that the flavonols HDG and KDG were present in flax, but no data were published pointing at their presence in an oligomeric structure.
Literature reported an HDG content of $0.01 \%$ (w/w) in flaxseed (Qiu et al., 1999), which is much lower than the $0.2 \%$ ( $\mathrm{w} / \mathrm{w}$ ) HDG in flaxseed hulls found in the present study. Flaxseed hulls might be a richer source of HDG compared to flaxseed, but also the extraction procedure used might explain the difference. Extraction under acidic conditions (Qiu et al., 1999) instead of under alkali conditions (this study) might lead to acid hydrolysis of HDG: deglucosylation (Qiu et al., 1999) takes place and a reactive herbacetin carbocation can be formed (Eklund et al., 2004). When water reacts with this carbocation, the less polar herbacetin can be formed. Because of the lower polarity of this herbacetin, it might be lost during the extraction procedure resulting in the lower yield. Alternatively, when organic solvents like MeOH or EtOH react with the carbocations, herbacetin ethers can be formed (Eklund et al., 2004). Qiu and coworkers (1999) indeed identified a herbacetin dimethylether. In the present study, upon full saponification, only HDG and no herbacetin or herbacetin ethers were found. So, both the loss of aglycons and the ether formation might explain the lower HDG content reported in literature in addition to the possible differences in HDG content between flaxseed and flaxseed hulls.
KDG, the other flavonol identified by Qiu and coworkers (1999), was not found in this study. Due to the low amount of sample material used, this was not expected.

Besides the release of HDG from the lignan macromolecule after alkali treatment, a fragment was isolated in which HDG was linked to HMGA, suggesting that HDG is part of the same macromolecular structure as lignans. This fragment was present as an ethylester, which was assumed to be formed as an intermediate product during saponification in the presence of ethanol (ethanolysis instead of hydrolysis) (Ford et al., 2001). Upon more severe saponification conditions the ethylesters were hydrolyzed again. During ethanolysis, transesterification between a macromolecular constituent and EtOH is assumed to take place. So, the identification of HDG+HMGA+EtOH suggests that at least one of the glucosyl residues of HDG is, via HMGA, ester-linked to another constituent of the macromolecule. Due to the low concentration of HDG it can not be excluded that fragments of HDG with 2 HMGA moieties are present. Therefore, it is still unclear whether in the lignan macromolecule HDG is present as constituent of the backbone or as a substituent.
The presence of HDG in the macromolecular structure is consistent with the biosynthesis of the lignan macromolecule. It has been proposed that SDG is incorporated in the lignan macromolecule by coupling of the glucosyl moieties with coenzyme A-activated HMGA (Ford et al., 2001). Since the glucosyl moieties and not the lignan itself are involved in the assembly of the macromolecule, it is hypothesized that also the glucosyl moieties of HDG could be a target of HMGA coupling.

Of all lignans described in flaxseed only SDG was found in this study. Because of varying concentrations of constituents due to variations in cultivar (Liggins et al., 2000) and growing conditions (Bhatty and Cherdkiatgumchai, 1990), and because of the use of flaxseed hulls instead of whole seeds, it was not expected that all compounds, especially those present in low concentrations in whole seeds, would be found in hulls. It was remarkable, however, that no MAT was found, MAT being the second most abundant lignan present in flaxseeds (Liggins et al., 2000; Mazur and Adlercreutz, 1998).
In analogy to the transformations described for hydroxymatairesinol (HMR) (Eklund et al., 2004), it is suggested that MAT is unstable under the alkaline conditions used in this study to obtain individual lignans. It is hypothesized that a reactive quinone was formed followed by the opening of the lactone-ring (Eklund et al., 2004). However, the expected reaction products were not identified by MS analysis. The browning of the reaction mixture, which was observed during saponification, might be attributed to quinone methide intermediates (Ekman et al., 1979) supporting the hypothesis of MAT quinone formation. In addition, it has been shown that lignan extraction without alkaline treatment could result in a higher yield of MAT (Milder et al., 2004). This indicates that the isolation of constituents from the lignan macromolecule is strongly dependent on the conditions applied.


Figure 6. The chemical structure of $\mathrm{HDG}+\mathrm{HMGA}+\mathrm{SDG}$; a structural element of the lignan macromolecule from flaxseed hulls.

In conclusion, the flavonol HDG is identified being present in flaxseed hulls as a subunit of a macromolecular structure, as shown in Fig. 6. HDG is incorporated in the macromolecule by HMGA, the same molecule that interconnects SDG. This shows that HMGA coupling is not limited to lignans, and suggests that more compounds carrying a glucosyl moiety might be incorporated in the lignan macromolecule.

## Experimental

## Lignan extraction from flaxseed hulls

Flaxseed hulls, kindly provided by Acatris Specialities Holding B.V. (Giessen, The Netherlands), were defatted by soxhlet extraction. In order to defat, 400 g of hulls were extracted with 3 l of $n$-hexane, resulting in 93.5 g oil extracted. The lignan macromolecule was extracted from the defatted hulls by a three-step sequential extraction with $63 \%$ ( $\mathrm{v} / \mathrm{v}$ ) aq. EtOH . In the first step, 9 ml of aq. EtOH per gram of defatted flaxseed hulls was used, in the second step $3.6 \mathrm{ml} / \mathrm{g}$ and in the last step $2.3 \mathrm{ml} / \mathrm{g}$. The first two extractions were performed for 4 hours at room temperature while stirring, the last extraction was performed overnight. The extracts and the hulls were separated by filtration on a 595 round paper filter (Schleicher \& Schuell). After evaporation of the EtOH, the extract was lyophilized yielding 29.9 g of lignan macromolecule.

## Saponification of the lignan macromolecule

Solutions of $2 \mathrm{mg} / \mathrm{ml}$ lignan macromolecule in $63 \%$ (v/v) aq. EtOH containing various concentrations NaOH were (partially) saponified to obtain lignan macromolecular fragments of various sizes. NaOH concentrations ranging from $1-25 \mathrm{mM} \mathrm{NaOH}$ were used for partial saponification. For full saponification 75 mM NaOH was used. Saponification was performed at room temperature while stirring. After 24 h , the reaction was stopped by lowering the pH to $6.5-7.0$ with HOAc ( $20 \mathrm{mM}-1.5 \mathrm{M}$ for analytical purposes, glacial HOAc for preparative purification purposes). For analytical purposes, the incubation volume was 1 ml , for preparative purification purposes a volume of 2.5 l was used.

## Sample clean up of saponified lignan macromolecule

Low molecular weight polar material was removed from the (partially) saponified samples by solid phase extraction (SPE; SepPak Vac, $20 \mathrm{cc} / 5 \mathrm{~g}$, C18 cartridge, Waters). Prior to loading samples onto the SPE cartridge, the EtOH concentration in the reaction mixture was reduced: the 1 ml samples were diluted with 25 ml water, whereas for the 2.5 l incubations the EtOH was evaporated and the remaining solution was concentrated further to max. 5 $\mathrm{mg} / \mathrm{ml}$. Insoluble particles formed during concentration, were removed by centrifugation ( $28,100 \mathrm{~g}, 4^{\circ} \mathrm{C}, 30 \mathrm{~min}$ ) and the supernatant was applied onto the cartridge. Resolubilisation of the insoluble material in $63 \%(\mathrm{v} / \mathrm{v})$ aq. EtOH and analysis of this material on analytical RP-HPLC did not show any peaks corresponding to peaks annotated as constituents of the lignan macromolecule (Fig. 3).
After activation of the SepPak cartridge with successively 2 column volumes of MeOH and 2 column volumes of water, a maximum of 150 mg (partially) saponified lignan macromolecule (supernatant) was loaded onto the cartridge. After a wash step with 1 column volume of water, lignans were eluted from the cartridge with 3 column volumes of MeOH of which the first column volume was discarded. For analytical purposes, the MeOH was evaporated under a stream of air to a final volume of 1 ml . For purification purposes, all MeOH was evaporated and the (partially) saponified lignans were lyophilized. Aliquots of the samples were analyzed on analytical RP-HPLC. After full and partial saponification

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( 2 mM NaOH ) of 5.0 g lyophilized lignan macromolecule and sample clean up, 2.5 g and 2.8 g of fully and partially saponified lignan macromolecule was obtained, respectively.

## Analytical reversed phase HPLC (RP-HPLC)

Samples were analyzed on an X-Terra C18 MS column (Waters; $3.5 \mu \mathrm{~m}$ particle size, 4.6 x 150 mm ) with an X-Terra C18 MS guard column (Waters; $3.5 \mu \mathrm{~m}$ particle size, $4.6 \times 10$ mm ) run on a Thermo Separation Products HPLC system equipped with a membrane degasser, P4000 pump, AS3000 autosampler, and UV3000 detector. Water and acetonitrile (ACN), both acidified with $0.1 \%(\mathrm{v} / \mathrm{v}) \mathrm{HOAc}$, were used as eluents. The flow rate was 0.7 $\mathrm{ml} / \mathrm{min}$. The following linear gradient was used: 0-25 min, 10-30\% ACN; 25-30 min, 30$50 \%$ ACN; 30-40 min, isocratic on $50 \%$ ACN; 40-42 min, 50-100\% ACN; 42-47 min, isocratic on $100 \% \mathrm{ACN}$; 47-50 min, $100-10 \% \mathrm{ACN}$; 50-60 min, isocratic on $10 \% \mathrm{ACN}$. The eluate was monitored at 280 nm . The injection volume was $20 \mu$ l. Samples were injected being solved in the solvent in which they were extracted or eluted (water, MeOH , $63 \%(\mathrm{v} / \mathrm{v})$ aq. EtOH or ACN:water mixtures).

## Preparative reversed phase HPLC

Lignans and fragments of the lignan macromolecule were purified by preparative RP-HPLC on an X-Terra C18 MS column (Waters; $5 \mu \mathrm{~m}$ particle size, $50 \times 100 \mathrm{~mm}$, OBD) with an XTerra C18 MS guard column (Waters; $5 \mu \mathrm{~m}$ particle size, $19 \times 10 \mathrm{~mm}$ ) ran on a Waters preparative HPLC system equipped with a 2525 pump, 2767 sample manager, Fluid Organizer and 2996 photodiode array detector. Water and ACN, both acidified with $0.01 \%$ TFA, were used as eluents. The flow rate was $80 \mathrm{ml} / \mathrm{min}$. The linear gradient was as follows: 0-16.6 min, 10-30\% ACN; 16.6-20 min, 30-50\% ACN; 20-26.6 min, isocratic on $50 \%$ ACN; 26.6-28 min, 50-100\% ACN; 28-31.3 min, isocratic on 100\% ACN; 31.3-33.3 $\min , 100-10 \%$ ACN; 33.3-40 min, isocratic on $10 \%$ ACN.
Up to 600 mg of lignans ( 2 mg partially or fully saponified lignan macromolecule/ ml water) were loaded per run. Prior to loading, the sample was filtered over a $0.2 \mu \mathrm{~m}$ cellulose acetate filter (Schleicher \& Schuell) to remove residual insoluble particles. Sample was loaded using the Waters reagent manager with a maximum flow rate of $2 \mathrm{ml} / \mathrm{min}$.
Based on the response at 280 nm , fractions were collected (leading edge gradient $=5 \%$, peak terminates when valley $=0$ ). Appropriate fractions were pooled, as were the corresponding pools from subsequent runs. ACN was evaporated and the fractions were lyophilized. The purity of the fractions pooled was determined on analytical HPLC.

## Semi-preparative reversed phase HPLC

For purification of 100 mg or less lignan macromolecule constituents, the samples were separated on a semi-preparative X-Terra C18 MS column (Waters; $5 \mu \mathrm{~m}$ particle size, 29 x 150 mm , OBD) with an X-Terra C18 MS guard column (Waters; $5 \mu \mathrm{~m}$ particle size, $19 \times 10$ mm ) ran on a Waters preparative HPLC system as specified above. The gradient was similar to the gradient of analytical RP-HPLC. The flow rate was $12 \mathrm{ml} / \mathrm{min}$. Fractions were collected based on the response at 280 nm as described for preparative RP-HPLC. The purity of the fractions was determined using analytical RP-HPLC and the purified components were lyophilized after evaporation of ACN.

## Liquid chromatography coupled on-line to mass spectrometry (LC-MS)

The molecular mass of the lignans and fragments of the macromolecule were determined on a Thermo Finnigan LCQ Classic coupled on-line to the analytical RP-HPLC. The flow from the analytical RP-HPLC was split: $1 / 10^{\text {th }}$ was directed to the MS. The MS was equipped with an ESI injector. Spectra were obtained in the negative ion mode over an $\mathrm{m} / \mathrm{z}$ range of $150-2000 \mathrm{Da}$. The capillary temperature was $270^{\circ} \mathrm{C}$, the capillary voltage was -7.00 V , the ion spray voltage was set on 4.50 kV and helium was used as sheath gas. MS/MS analysis was performed with a normalized collision energy of $27 \%$.

## Gel permeation chromatography (GPC)

To determine the molecular weight distribution of the lignan macromolecule and the saponified lignans, aliquots of $50 \mu \mathrm{l}(2 \mathrm{mg} / \mathrm{ml})$ were analyzed on a similar HPLC system as described for analytical RP-HPLC, equipped with a Tricorn Superdex Peptide 10/300 GL column (Amersham Bioscience; $10 \times 300-310 \mathrm{~mm}$, bed volume $=24 \mathrm{ml}$, optimum separation range $100-7000 \mathrm{Da}$ ). The column was run isocratically at a flow rate of 0.8 $\mathrm{ml} / \mathrm{min}$ with $40 \%(\mathrm{v} / \mathrm{v})$ aq. $\mathrm{ACN}+0.1 \%(\mathrm{v} / \mathrm{v})$ TFA as eluent. The eluate was monitored at 280 nm . The included volume ( $\mathrm{V}_{\mathrm{inc}}=16.5 \mathrm{ml}$ ) was determined with water. It should be noted that part of the compounds eluted later than the included volume indicating that aspecific interactions occurred. No further calibration was performed since no suitable calibration compounds were available.

## Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDITOF MS)

For the qualification of the molecular weight distribution of unsaponified lignan macromolecule, this sample was analyzed on a Ultraflex MALDI-TOF MS (Bruker Daltronics GmbH). As matrix 2,5-dihydroxybenzoic acid dissolved in 50\% aq. ACN (15 $\mathrm{mg} / \mathrm{ml}$ ) was used. $2 \mu \mathrm{l}$ of sample mixture consisting of $10 \mu \mathrm{l} 2 \mathrm{mg} / \mathrm{ml}$ unsaponified ( 0 mM ) lignan macromolecule, $10 \mu \mathrm{l}$ matrix-solution and $1 \mu \mathrm{l} 1 \mathrm{mM} \mathrm{NaAc} \mathrm{pH} 5$ was spotted on a gold plate. The MALDI-TOF MS was calibrated with a mixture of malto-dextrins (mass range 365-4092). The system was used in the positive reflector mode.

## Nuclear magnetic resonance (NMR)

Prior to NMR analyses, the samples were solved in $99.8 \% \mathrm{CD}_{3} \mathrm{OD}+0.05 \%$ (v/v) TMS (Cambridge Isotope Laboratories, USA). NMR spectra were recorded at a probe temperature of $25^{\circ} \mathrm{C}$ on a Bruker AMX-500 spectrometer located at the Wageningen NMR Centre. Chemical shifts were expressed in ppm relative to TMS at 0.00 ppm .
The ${ }^{1} \mathrm{H}$ and ${ }^{13} \mathrm{C}$ proton decoupled spectra were recorded at 500.13 MHz and 125.77 Hz , respectively. All 2D COSY spectra were acquired using the double quantum filtered (DQF) method with a standard pulse sequence delivered by Bruker.
For the 2D HMBC spectrum a standard gradient enhanced 2D HMQC pulse sequence delivered by Bruker, was changed into a HMBC sequence by setting the delay between the first proton and carbon pulse to 50 ms .

## Spectral data of constituents of the lignan macromolecule

trans-p-Coumaric acid $\beta$-glucopyranoside (1): ${ }^{1} \mathrm{H}$ NMR ( $500.13 \mathrm{MHz}, \mathrm{CD}_{3} \mathrm{OD}$ ) $\delta 7.55$ ( 2 H , $d, J=8.61 \mathrm{~Hz}, \mathrm{H}-2 / 6), 7.12(2 \mathrm{H}, d, J=8.56 \mathrm{~Hz}, \mathrm{H}-3 / 5)$, $7.63(1 \mathrm{H}, d, J=15.94 \mathrm{~Hz}, \mathrm{H}-7), 6.36$ $(1 \mathrm{H}, d, J=15.94 \mathrm{~Hz}, \mathrm{H}-8), 4.97\left(1 \mathrm{H}, d, J=6.68 \mathrm{~Hz}, \mathrm{H}-1^{\prime}\right), 3.46$ ( $\left.3 \mathrm{H}, m, \mathrm{H}-2^{\prime} / 3^{\prime} / 5^{\prime}\right), 3.40$ ( $1 \mathrm{H}, m, \mathrm{H}-4$ '), 3.90 ( $1 \mathrm{H}, d d, J=12.14,1.46 \mathrm{~Hz}, 1 \mathrm{H}, \mathrm{H}-6 \mathrm{a}$ ), 3.70 ( $1 \mathrm{H}, d d, J=12.06,5.52$ Hz, 1 H, H6b’).
${ }^{13} \mathrm{C}$ NMR ( $125.77 \mathrm{MHz}, \mathrm{CD}_{3} \mathrm{OD}$ ) $\delta 130.0$ (C-1), 130.9 (C-2 and 6), 117.8 (C-3 and 5), 160.9 (C-4), 146.1 (C-7), 117.4 (C-8), 170.4 (COOD), 101.7 (C-1'), 74.8 (C-2'), 77.8 (C3'), 71.3 (C-4'), 78.2 (C-5'), 62.6 (C-6').
trans-Ferulic acid $\beta$-glucopyranoside (2): ${ }^{1} \mathrm{H}$ NMR ( $500 \mathrm{MHz}, \mathrm{CD}_{3} \mathrm{OD}$ ) $\delta 7.61$ ( $1 \mathrm{H}, d$, $J=15.93 \mathrm{~Hz}, \mathrm{H}-7$ ), 7.24 ( $1 \mathrm{H}, d, J<1 \mathrm{~Hz}, \mathrm{H}-2$ ), 7.15 ( $1 \mathrm{H}, d d, J=8.46,1.50 \mathrm{~Hz}, \mathrm{H}-6$ ), 7.18 (1 $\mathrm{H}, d, J=8.37 \mathrm{~Hz}, \mathrm{H}-5), 6.39(1 \mathrm{H}, d, J=15.92 \mathrm{~Hz}, \mathrm{H}-8), 4.97(1 \mathrm{H}, d, J=7.38 \mathrm{~Hz}, \mathrm{H}-1$ '), 3.90 ( $1 \mathrm{H}, \mathrm{s}, \mathrm{CH}_{3}$ ), $3.88(1 \mathrm{H}, d d, J=12.06,1.89 \mathrm{~Hz}, \mathrm{H}-6 \mathrm{a}$ '), $3.69(1 \mathrm{H}, d d, J=12.06,5.21 \mathrm{~Hz}$, H6b’).
${ }^{13} \mathrm{C}$ NMR ( $125.77 \mathrm{MHz}, \mathrm{CD}_{3} \mathrm{OD}$ ) $\delta 130.5$ (C-1), 112.5 (C-2), $151.0(\mathrm{C}-3), 149.9$ (C-4), 117.3 (C-5), 123.6 (C-6) 146.2 (C-7), 117.9 (C-8), 170.5 (COOD), $56.6\left(\mathrm{CH}_{3}\right), 102.3$ (C-1’), 74.9 (C-2'), 77.9 (C-3'), 71.4 (C-4'), 78.4 (C-5'), 62.5 (C-6’).

Secoisolariciresinol-di- $\beta$-glucopyranoside (4): ${ }^{1} \mathrm{H}$ NMR ( $500.13 \mathrm{MHz}, \mathrm{CD}_{3} \mathrm{OD}$ ) $\delta 6.58$ (2 H, s, H-2/2’), 6.65 ( $2 \mathrm{H}, d, J=7.9 \mathrm{~Hz}, \mathrm{H}-5 / 5$ '), 6.56 ( $2 \mathrm{H}, d, J=7.98 \mathrm{~Hz}, \mathrm{H}-6 / 6$ ’), 2.69 ( 2 H , $d d, J=13.72,6.58 \mathrm{~Hz}, \mathrm{H}-7 \mathrm{a} / 7 \mathrm{a}$ '), 2.61 ( $2 \mathrm{H}, d d, J=13.62,8.07 \mathrm{~Hz}, \mathrm{H} 7 \mathrm{~b} / 7 \mathrm{~b}$ '), 2.12 ( $2 \mathrm{H}, m$, H-8/8'), 4.08 ( $2 \mathrm{H}, d d, J=9.83,5.50 \mathrm{~Hz}, \mathrm{H}-9 \mathrm{a} / 9 \mathrm{a}$ '), 3.47 ( $2 \mathrm{H}, d d, J=9.75,6.56 \mathrm{~Hz}, \mathrm{H}-$ 9b/1b'), 3.73 ( $6 \mathrm{H}, \mathrm{s}, \mathrm{OCH}_{3}$ ), 4.24 ( $2 \mathrm{H}, d, \mathrm{~J}=7.79 \mathrm{~Hz}, \mathrm{H}-1$ '’/1’’’), 3.215 ( $2 \mathrm{H}, \mathrm{H}-2$ '’/2’’'),
 $J=11.80,1.83 \mathrm{~Hz}, \mathrm{H}-6 \mathrm{a}$ '/ $/ 6 \mathrm{a}$ '"'), 3.69 ( $2 \mathrm{H}, d d, J=11.84,5.54 \mathrm{~Hz}, \mathrm{H}-6 \mathrm{~b} ’$ '/6b'’').
${ }^{13} \mathrm{C}$ NMR (125.77 MHz, CD ${ }_{3} \mathrm{OD}$ ) $\delta 134.0$ (C-1/1'), 113.6 (C-2/2'), 148.8 (C-3/3'), 145.4 (C-4/4'), 115.7 (C-5/5'), 122.9 (C-6/6'), 35.7 (C-7/7’), 41.3 (C-8/8'), 71.2 (C-9/9'), 104.8 (C-1'’/1'’'), 75.3 (C-2'’/2'’'), 78.2 (C-3’’/3'’’), 71.7 (C-4'’/4’’'), 77.9 (C-5’'/5'’'), 62.9 (C6"'/6'’').

## References

Bakke, J.E., Klosterman, H.J., 1956. A new diglucoside from flaxseed. Proc. North Dakota Acad. Sci. 10, 18-21.

Bhatty, R.S., Cherdkiatgumchai, P., 1990. Compositional analysis of laboratory-prepared and commercial samples of linseed meal and of hull isolated from flax. J. Am. Oil Chem. Soc. 67, 79-84.

Bhatty, R.S., 1995. Nutrient composition of whole flaxseed and flaxseed meal. In: Cunnane, S.C.,Thompson, L.U. (Eds.), Flaxseed in Human Nutrition. AOCS Press, Champaign, Illinois, pp. 22-42.

Boccardo, F., Lunardi, G., Guglielmini, P., Parodi, M., Murialdo, R., Schettini, G., Rubagotti, A., 2004. Serum enterolactone levels and the risk of breast cancer in women with palpable cysts. Eur. J. Cancer 40, 84-89.

Chen, J.M., Stavro, P.M., Thompson, L.U., 2002. Dietary flaxseed inhibits human breast cancer growth and metastasis and downregulates expression of insulin-like growth factor and epidermal growth factor receptor. Nutr. Cancer 43, 187-192.

Chen, J.M., Thompson, L.U., 2003. Lignans and tamoxifen, alone or in combination, reduce human breast cancer cell adhesion, invasion and migration in vitro. Breast Cancer Res. Treat. 80, 163-170.

Dabrowski, K.J., Sosulski, F.W., 1984. Composition of free and hydrolyzable phenolic acids in defatted flours of ten oilseeds. J. Agric. Food Chem. 32, 128-130.

Eklund, P.C., Sundell, F.J., Smeds, A.I., Sjoholm, R.E., 2004. Reactions of the natural lignan hydroxymatairesinol in basic and acidic nucleophilic media: formation and reactivity of a quinone methide intermediate. Org. Biomol. Chem. 2, 2229-2235.

Ekman, R., Sjoholm, R.T., Sjoholm, R., 1979. Degraded lignan from alkaline-hydrolysis of Norway spruce root extractives. Finnish Chem. Lett. 126-128.

Ford, J.D., Huang, K.S., Wang, H.B., Davin, L.B., 2001. Biosynthetic pathway to the cancer chemopreventive secoisolariciresinol diglucoside-hydroxymethyl glutaryl esterlinked lignan oligomers in flax (Linum usitatissimum) seed. J. Nat. Prod. 65, 1388-1397.

Johnsson, P., Kamal-Eldin, A., Lundgren, L.N., Åman, P., 2000. HPLC method for analysis of secoisolariciresinol diglucoside in flaxseeds. J. Agric. Food Chem. 48, 5216-5219.

Johnsson, P., Peerlkamp, N., Kamal-Eldin, A., Andersson, R.E., Andersson, R., Lundgren, L.N., Aman, P., 2002. Polymeric fractions containing phenol glucosides in flaxseed. Food Chem. 76, 207-212.

Kamal-Eldin, A., Peerlkamp, N., Johnsson, P., Andersson, R., Andersson, R.E., Lundgren, L.N., Aman, P., 2001. An oligomer from flaxseed composed of secoisolariciresinoldiglucoside and 3-hydroxy-3-methyl glutaric acid residues. Phytochemistry 58, 587-590.

Klosterman, H.J., Smith, F., 1954. The isolation of beta-hydroxy-beta-methylglutaric acid from the seed of flax (Linum-usitatissimum). J. Am. Chem. Soc. 76, 1229-1230.

Klosterman, H.J., Smith, F., Clagett, C.O., 1955. The constitution of linocinnamarin. J. Am. Chem. Soc. 77, 420-421.

Liggins, J., Grimwood, R., Bingham, S.A., 2000. Extraction and quantification of lignan phytoestrogens in food and human samples. Anal. Biochem. 287, 102-109.

Lucas, E.A., Lightfoot, S.A., Hammond, L.J., Devareddy, L., Khalil, D.A., Daggy, B.P., Smith, B.J., Westcott, N., Mocanu, V., Soung, D.Y., Arjmandj, B.H., 2004. Flaxseed reduces plasma cholesterol and atherosclerotic lesion formation in ovariectomized Golden Syrian hamsters. Artherosclerosis 173, 223-229.

Madhusudhan, B., Wiesenborn, D., Schwarz, J., Tostenson, K., Gillespie, J., 2000. A dry mechanical method for concentrating the lignan secoisolariciresinol diglucoside in flaxseed. Food. Sci. Technol. 33, 268-275.

Mazur, W., Adlercreutz, H., 1998. Naturally occurring oestrogens in food. Pure Appl. Chem. 70, 1759-1776.

Meagher, L.P., Beecher, G.R., Flanagan, V.P., Li, B.W., 1999. Isolation and characterization of the lignans, isolariciresinol and pinoresinol, in flaxseed meal. J. Agric. Food Chem. 47, 3173-3180.

Milder, I.E.J., Arts, L.C.W., Venema, D.P., Lasaroms, J.J.P., Wahala, K., Hollman, P.C.H., 2004. Optimization of a liquid chromatography-tandem mass spectrometry method for quantification of the plant lignans secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol in foods. J. Agric. Food Chem. 52, 4643-4651.

Milder, I.E.J., Arts, I.C.W., van de Putte, B., Venema, D.P., Hollman, P.C.H., 2005. Lignan contents of Dutch plant foods: a database including lariciresinol, pinoresinol, secoisolariciresinol and matairesinol. Br. J. Nutr. 93, 393-402.

Oomah, B.D., Mazza, G., 1997. Effect of dehulling on chemical composition and physical properties of flaxseed. Food Sci. Technol.-Lebensm.-Wiss. Technol. 30, 135-140.

Oomah, B.D., 2001. Flaxseed as a functional food source. J. Sci. Food Agric. 81, 889-894.
Qiu, S.-X., Lu, Z.-Z., Luyengi, L., Lee, S.K., Pezzuto, J.M., Farnsworth, N.R., Thompson, L.U., Fong, H.H.S., 1999. Isolation and characterization of flaxseed (Linum usitatissimum) constituents. Pharm. Biol. 37, 1-7.

Sicilia, T., Niemeyer, H.B., Honig, D.M., Metzler, M., 2003. Identification and stereochemical characterization of lignans in flaxseed and pumpkin seeds. J. Agric. Food Chem. 51, 1181-1188.

Sung, M.K., Lautens, M., Thompson, L.U., 1998. Mammalian lignans inhibit the growth of estrogen-independent human colon tumor cells. Anticancer Res. 18, 1405-1408.

Thompson, L.U., Rickard, S.E., Orcheson, L.J., Seidl, M.M., 1996a. Flaxseed and its lignan and oil components reduce mammary tumor growth at a late stage of carcinogenesis. Carcinogenesis 17, 1373-1376.

Thompson, L.U., Seidl, M.M., Rickard, S.E., Orcheson, L.J., Fong, H.H.S., 1996b. Antitumorigenic effect of a mammalian lignan precursor from flaxseed. Nutr. Cancer 26, 159-165.

Vanharanta, M., Voutilainen, S., Lakka, T.A., van der Lee, M., Adlercreutz, H., Salonen, J.T., 1999. Risk of acute coronary events according to serum concentrations of enterolactone: a prospective population-based case-control study. Lancet 354, 2112-2115.

Wiesenborn, D., Tostenson, K., Kangas, N., 2003. Continuous abrasive method for mechanically fractionating flaxseed. J. Am. Oil Chem. Soc. 80, 295-300.

# Chapter 3 <br> Hydroxycinnamic acids are ester-linked directly to glucosyl moieties within the lignan macromolecule from flaxseed hulls 




#### Abstract

In flaxseed hulls, lignans are present in an oligomeric structure. Secoisolariciresinol diglucoside (SDG), ester linked to hydroxy-methyl-glutaric acid (HMGA), forms the backbone of this lignan macromolecule. The hydroxycinnamic acids p-coumaric acid glucoside (CouAG) and ferulic acid glucoside (FeAG), are also part of the lignan macromolecule. However, their position and type of linkage are still unknown. The aim of this study was to investigate how CouAG and FeAG are linked within the lignan macromolecule from flaxseed hulls. Fragments of the lignan macromolecule were obtained by partial saponification. After isolation of the fragments by preparative RP-HPLC, several key structures were identified by MS and NMR. Within the lignan macromolecule, CouAG is attached to the C-6 position of a glucosyl moiety of SDG. FeA is linked to the C-2 position of a glucosyl moiety of SDG. FeAG is ester-linked within the lignan macromolecule with its carboxylic group, but it remains unclear whether FeAG links to the C-2 or C-6 position of SDG. Attachment of HMGA to the glucosyl moiety of CouAG or FeAG was not observed. The results clearly show that within the lignan macromolecule, the hydroxycinnamic acids are linked directly via an ester bond to the glucosyl moiety of SDG.


## Introduction

Lignans are phenolic compounds, which are widely distributed in plants. Especially in seeds and nuts, high concentrations can be found (Milder et al., 2005). Concentrations up to $3 \%(\mathrm{w} / \mathrm{w})$ have been reported in flaxseeds, making flax one of the richest edible sources of lignans (Eliasson et al., 2003; Milder et al., 2005). Secoisolariciresinol diglucoside (SDG) is the most important lignan in flaxseed (Milder et al., 2005). After ingestion, SDG is converted into the mammalian lignans enterodiol and enterolacton, which exhibit several health beneficial effects (Thompson et al., 1996; Vanharanta et al., 1999; Ward et al., 2001).

In contrast to most plants in which free lignans are present, the lignans in flaxseeds are incorporated into an oligomeric structure (Kamal-Eldin et al., 2001; Westcott and Muir, 1996), which is referred to as lignan macromolecule. This lignan macromolecule is also reported in flaxseed hulls (Struijs et al., 2007).
Although a variety of lignans (Bakke and Klosterman, 1956; Liggins et al., 2000; Meagher et al., 1999; Sicilia et al., 2003), flavonoids (Qiu et al., 1999), and (hydroxy)cinnamic acids (Dabrowski and Sosulski, 1984; Klosterman et al., 1955; Westcott and Muir, 1996) have been identified in flaxseed extracts, only a small number of these constituents are related to the lignan macromolecule. The most abundant lignan in flaxseeds, secoisolariciresinol diglucoside (SDG), is esterified to hydroxy-methyl-glutaric acid (HMGA), thereby forming the backbone of the lignan macromolecule (Kamal-Eldin et al., 2001). p-Coumaric acid glucoside (CouAG) and ferulic acid glucoside (FeAG) are thought to be part of the lignan macromolecule, since they are only observed after alkali treatment of flaxseed extracts containing lignan macromolecule (Johnsson et al., 2002; Struijs et al., 2007; Westcott and Muir, 1996). Also the release of caffeic acid glucoside from a similar extract has been reported (Westcott and Muir, 1996). Recently, it was shown that the flavonoid herbacetin diglucoside (HDG) is also part of the macromolecule (Struijs et al., 2007). In a similar way as SDG, HDG is attached within the lignan macromolecule via ester-linkages with HMGA. Until now, it is unclear how the hydroxycinnamic acid glucosides are linked within the lignan macromolecule. In-line with the attachment of SDG and HDG, hydroxycinnamic acid glucosides might be linked within the lignan macromolecule via ester-linkage of their glucosyl moieties to HMGA. Another possibility is that they are ester-linked directly with their carboxyl group to a glucosyl moiety of SDG.
The aim of the present study is to identify the linkage of the hydroxycinnamic acid glucosides CouAG and FeAG within the lignan macromolecule from flaxseed hulls.

## Results

For the isolation of structural elements of the lignan macromolecule from flaxseed hulls containing CouAG or FeAG, the procedure of partial saponification was followed as previously described (Struijs et al., 2007). It should be noted that during saponification, transesterification between ethanol and the lignan macromolecule can take place so that ethanolates can be formed (Ford et al., 2001; Johnsson et al., 2002).
Fragments of the lignan macromolecule formed during partial saponification, were separated by preparative RP-HPLC. Fractions were collected as indicated by the marks in the RP-HPLC profile (Fig. 1). All fractions were analyzed on analytical RP-HPLC/MS. The MS-data were screened for $m / z$-ratios corresponding to fragments composed of monomeric constituents of the lignan macromolecule being p-coumaric acid (CouA), CouAG, ferulic acid (FeA), FeAG, HDG, secoisolariciresinol (SECO), SDG, HMGA, their ethanolates and combinations thereof. Only those fractions with high signal to noise ratios on MS, high UVsignals, or annotations matching the previously mentioned compositional criteria, were studied further. On this basis, eleven fractions were selected as indicated by the numbers 1 to 11 in Fig. 1.


Figure 1. Preparative RP-HPLC profile of partially saponified lignan macromolecule. The collected fractions are indicated. Annotations of the numbered peaks are listed in Table 1.

In Table 1 the $m / z$-ratios of the fractions 1 to 11 with their corresponding annotations are listed. Also the MS/MS data were supporting for these annotations. The number of compounds present in each fraction might be overestimated, as peaks were overlapping (Fig. 1) and fragmentation as a result of ionization during MS analysis could occur.

Table 1. Annotations of the fragments obtained by partial saponification of the lignan macromolecule as determined by RP-HPLC/MS (negative mode) and MS/MS detection.

| Fraction | Annotation | $\mathrm{m} / \mathrm{z}[\mathrm{M}-\mathrm{H}]^{-}$ | MS/MS |
| :---: | :---: | :---: | :---: |
| 1 | CouAG | 325.0 | 163.1; 204.8 |
| 2 | FeAG | 355.0 | 178.1; 193.0 |
| 3 | HDG | 625.2 | 463.1 |
| 4 | SDG | 685.3 | 361.4; 523.2 |
|  | HDG+HMGA | 769.2 | 463.1; 607.1; 625.2; 707.1 |
| 5 | SDG+HMGA*\# | 829.1 | 523.3; 667.1; 685.1 |
| 6 | SDG+HMGA* | 829.1 | 523.1; 667.2; 685.2 |
|  | HDG + $\mathrm{HMGA}+\mathrm{EtOH}^{*}$ | 797.2 | 463.1; 505.2; 625.2; 667.1 |
|  | SDG+CouA | 831.2 | 523.3; 667.2; 686.2 |
|  | SDG+2HMGA* | 973.2 | 685.2; 829.2; 871.1 |
|  | SDG+CouAG | 993.0 | 709.0 |
|  | SDG+HMGA+CouAG* | 1137.1 | ND |
| 7 | SDG+2HMGA | 973.2 | 685.3; 829.1; 871.1 |
|  | HDG+HMGA+EtOH | 797.2 | 463.2; 607.2; 625.2; 779.0 |
|  | SDG+HMGA | 829.2 | 667.2; 685.2; 727.0 |
|  | SDG+HMGA+CouAG | 1137.2 | 974.8 |
|  | SDG+HMGA+FeAG | 1167.2 | 861.1; 1004.9 |
|  | SDG+HMGA+HDG | 1437.3 | 463.1; 625.2; 667.2; 1275.3 |
| 8 | SDG+2HMGA* | 973.1 | 685.2; 829.1; 871.1 |
|  | SDG+FeA*\# | 861.1 | 343.3; 505.1; 667.1; 685.1 |
|  | SDG+HMGA+CouAG* | 1137.2 | 831.2; 873.2; 913.2; 975.2 |
|  | SDG+HMGA+FeAG* | 1167.2 | 861.2; 1005.1 |
|  | SDG+HMGA+HDG | 1437.2 | 463.0; 667.1; 1275.1 |
|  | SDG+2HMGA+HDG* | 1581.0 | ND |
| 9 | CouAG+EtOH+acetate* | 412.9 | 191.1; 250.1; 352.7 |
|  | SDG+FeA+520?* | 1363.1 | 861.2; 1057.1; 1219.1 |
|  | HDG+HMGA+EtOH* | 797.1 | ND |
|  | SDG + FeA* | 861.2 | 523.2; 667.2; 685.2 |
|  | SDG+HMGA+CouAG | 1137.1 | 614.6 |
|  | SDG+CouAG+FeA* | 1169.0 | 831.2; 861.3; 1007.3 |
|  | SDG+2HMGA+CouAG* | 1281.3 | 831.1; 975.1; 1119.2; 1137.2 |
| 10 | SDG+HMGA+FeA* | 1005.1 | 829.3; 861.2 |
|  | FeAG+EtOH+acetate* | 442.9 | 221.1; 281.0; 382.5 |
|  | SDG+FeA*\# | 861.2 | 505.2; 667.1; 685.0 |
|  | SDG + HMGA+CouA* | 975.1 | 667.1; 829.0; 831.2; 873.2 |
| 11 | SDG+2HMGA+EtOH | 1001.1 | 685.4; 727.3; 973.3 |
|  | 2SDG+HMGA | 1497.1 | ND |
|  | 2SDG+2HMGA | 1641.0 | ND |
|  | SDG+CouA | 831.2 | 325.1; 523.2; 667.2; 685.2 |
|  | SDG+FeA | 861.1 | 361.2; 505.3; 523.2; 699.2 |

Fractions 5, 6, 8, 9, and 10 were subjected to semi-preparative separation. Fragments indicated with * were found in both preparative and semi-preparative separations. MS/MS data were collected after preparative separation. MS/MS data collected after semi-preparative separation are marked with \#. Bold numbers are the predominant $m / z$-ratios. ND $=$ no MS/MS data available.? The $m / z$-ratio of 861.2 $\left[\mathrm{M}-\mathrm{H}^{+}\right]^{-}$in the MS/MS spectrum of the fragment with an $m / z$-ratio of $1363.1\left[\mathrm{M}-\mathrm{H}^{+}\right]^{-}$points at the presence of SDG +Fe A in this fragment. The rest of the fragment remained unidentified.

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Some fragments were solely annotated based on their $m / z$-ratios (no MS/MS data obtained), but the likeliness of the presence of these fragments allowed annotation as such.
In accordance with the identification of the monomeric constituents after full saponification (Struijs et al., 2007), fractions 1 to 4 were annotated as the monomeric constituents of the lignan macromolecule. In fractions 5 to 8 , high intensity $\mathrm{m} / \mathrm{z}$-ratios corresponding to fragments consisting of SDG and HMGA were found. In fractions 6 to 11, fragments of the lignan macromolecule with $m / z$-ratios corresponding to $\operatorname{SDG}$ plus $\operatorname{FeA}(\mathrm{G})$ or CouA(G), and SDG+HMGA plus $\mathrm{Fe}(\mathrm{A}(\mathrm{G})$ or $\mathrm{CouA}(\mathrm{G})$ were annotated. Especially these fragments were of interest for the identification of linkage types between SDG and CouA(G) or FeA(G). Therefore, they were analyzed further by NMR. A fragment of HDG+HMGA+EtOH, which shows the linkage of HDG within the lignan macromolecule, has been described before (Struijs et al., 2007).

Since the fractions putatively consisting of SDG and hydroxycinnamic acids were not base line separated, fractions $5,6,8,9$, and 10 were subjected to a semi-preparative purification step before NMR analysis. After semi-preparative purification, fractions $5^{*}, 6^{*}, 8^{*}, 9^{*}$, and 10* were obtained, and analyzed on analytical RP-HPLC/MS. The abundance of fraction 7 was found to be too low and fraction 11 too complex for further purification.
The analytical RP-HPLC profiles of the five fractions obtained by semi-preparative RPHPLC, showed that, except for some small shoulders, the peaks were pure based on HPLCUV signals (data not shown). However, MS analysis revealed several $\mathrm{m} / \mathrm{z}$-values per fraction. In Table 1, the putative compounds, which were still present after semi-preparative purification, are indicated with an asterisk (*). It is remarkable that despite the semipreparative purification, several $m / z$-ratios (e.g. $m / z=829.1\left[M-H^{+}\right]^{-}$and $m / z=1137.2[\mathrm{M}-$ $\left.\mathrm{H}^{+}\right]^{-}$) were found back in several fractions. No further attempts to obtain more pure fractions were performed. The five fractions purified by semi-preparative RP-HPLC were analyzed by NMR to confirm the annotations made based on MS-data and to identify the linkage types between the hydroxycinnamic acids and SDG. In Table 2 the structural elements identified by NMR are listed, including the numbering used throughout the text.

In fraction 5* based on the COSY and HMBC spectra, the structural element SDG+HMGA was identified. Chemical shifts corresponded to those of SDG and HMGA reported in literature (Kamal-Eldin et al., 2001; Struijs et al., 2007). Besides, a cross-peak between C- $\alpha$ and $\mathrm{H}-6 \mathrm{a} \mathrm{a} / \mathrm{b}$ corresponded to the coupling of the carboxyl group of HMGA to one of the glucosyl units of SDG. Neither the C- $\varepsilon$ nor the C- 6 "' showed up- or downfield shifts of proton and carbon signals, nor a cross-peak indicating a linkage. This showed that the HMGA also carried a free carboxyl-group ( $\mathrm{C}-\varepsilon$ ) and that the second glucosyl moiety of SDG had a free C-6 position (C-6"'). These data corresponded to the structural element of SDG+HMGA as shown in Table 2.

The HMBC spectrum of fraction 6* is shown in Fig. 2. Two structures were identified in this fraction. The first structure identified in the HMBC spectrum was HDG+HMGA+EtOH, as identified before (Struijs et al., 2007). Second, a structural element consisting of SDG+CouAG was found. The chemical shifts of this fragment are given in Table 3. The hydroxyl group of CouA linked via a glycosidic bond to glucose,
showing the presence of CouAG (in Fig. 2 cross-peak indicated as CouA 4*, Glc 1"'). The chemical shifts of this coupling were as reported in literature (Johnsson et al., 2002; Struijs et al., 2007). The carboxyl-group of CouAG (C-9*) coupled to the C-6" of a glucosyl residue of SDG as shown by a cross-peak between C-9* and the downfield shifted H-6"a/b (in Fig. 2 cross-peak indicated as CouA 9, Glc 6"a/b). Cross-peaks indicating linkage of CouAG to another position of SDG were not found. These results showed that CouAG is linked with its carboxyl group to the C-6 position of a glucosyl moiety of SDG (see Table 2). Also in fraction $8^{*}$ some low intensity cross-peaks indicating SDG+CouAG were present. No indications for linkage of CouA to another constituent were obtained by NMR. The annotation of fragments containing CouA based on MS analysis are most likely explained by occurrence of fragmentation during MS analysis.

Table 2. Structural elements of the lignan macromolecule identified by NMR.


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(Table 2. continued)






10* $\mathrm{FeAG}+\mathrm{EtOH}$


Based on the proton-NMR spectra of fraction 9* it was confirmed that CouAG is linked within the macromolecule via its carboxylic group. This fraction consisted predominantly of CouAG + EtOH (see Table 2). The ethanol-ester was present on the carboxyl group (C-9) of

CouAG as shown by the slight upfield shift of H-8. Besides, the chemical shifts of the ethanolate group were comparable to the ones found for $\mathrm{FeAG}+\mathrm{EtOH}$ as described later. Furthermore, the integrals of all proton signals of this spin system fitted well with the proposed structure (see spectral data in section 4).
A fragment annotated based on the MS data as SDG+HMGA+CouAG (Table 1, fraction 6, 7, and 8), suggested that the structural element of SDG+CouAG, as identified by NMR, could be elongated by HMGA. However, the MS/MS data (Table 1) were not conclusive about which glucosyl moiety carried HMGA.


Figure 2. HMBC spectrum of fraction 6* of the lignan macromolecule. Cross-peaks of SDG+CouAG (CouAG and linkage to glucose: - ; SDG: $\cdots \cdots \cdot$ ), and HDG+HMGA+EtOH (-----) are indicated. Numbering corresponds to numbers given in Table 2. Cross-peaks are indicated as the coupling of carbon to proton. For example, the notation of CouA 9*, Glc 6" indicates the coupling of the C-9* to the $\mathrm{H}-6$ ", the notation Glc"' indicates a single bond coupling of $\mathrm{C}-1$ "' to $\mathrm{H}-1$ "' of Glc. Due to overlapping signals, it could not be determined to which compound Glc1** (-----) is attached.

In fraction 8*, cross-peaks pointing at the presence of two main structural elements were identified: SDG+2HMGA, and SDG+FeA (see Fig. 3 for HMBC spectra, Table 3 for chemical shifts, and Table 2 for the chemical structures). For the structural element of SDG+2HMGA, again chemical shifts corresponding to SDG and HMGA were observed. A

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cross-peak between $\mathrm{C}-\alpha$ and $\mathrm{H}-6$ 'a/b was present in the HMBC spectrum showing the coupling between the carboxyl group of HMGA and the C-6 of the glucosyl residue of SDG (in Fig. 3, cross-peak indicated as HMGA $\alpha$, Glc 6'a/b). The structural element SDG+2HMGA (see Table 2) was identified since its spin system showed only one glucose anomeric signal (Glc C-1') with downfield shifted H-6'a/b protons, both showing crosspeaks in the HMBC spectrum to the C- $\alpha$ of HMGA. Additional proof for this symmetrical molecule was the slightly upfield shifted H-9a compared to a glucosyl residue of SDG which is not substituted with HMGA.


Figure 3. HMBC spectrum of fraction 8* of the lignan macromolecule. Cross-peaks of SDG+2HMGA ( $\cdots \cdots$ ), SDG+FeA (-----) and SDG+CouAG ( - ) are indicated. Numbering corresponds to numbers given in Table 2. Cross-peaks are indicated as the coupling of carbon to proton. For example, the notation of FeA 9*, Glc 2" indicates the coupling of the C-9* to the H-2", the notation Glc"' indicates a single bond coupling of $\mathrm{C}-1$ "' to $\mathrm{H}-1$ "' of Glc.

In fraction 8*, also chemical shifts corresponding to FeA were found (see Table 3). In the HMBC spectrum (Fig. 3) no coupling of glucose to the C-4* position of FeA was observed. The coupling of SDG to FeA was identified by a cross-peak between C-9* and H-2" showing an ester-linkage between the carboxyl group of FeA and the $\mathrm{C}-2$ position of a glucosyl residue of SDG (in Fig. 3, cross-peak indicated as FeA 9*, Glc 2"). The identification of a structural element with FeA was surprising, since in the RP-HPLC/MS data of the fully saponified lignan macromolecule only very low intensity $\mathrm{m} / \mathrm{z}$-values annotated as $\mathrm{FeA}+\mathrm{EtOH}$ were found (data not shown).

Also in fraction $10^{*}$, some low intensity peaks were present indicating the presence of SDG + FeA with FeA linked to the C-2 position of glucose. In agreement with these weak NMR signals was the annotation of the fragment of SDG $+\mathrm{HMGA}+\mathrm{Fe}$ A based on MS results of fraction $10^{*}$. The MS/MS data showed that FeA or HMGA was split off, so that both FeA and HMGA formed a terminal group of this fragment. In the HMBC spectrum of fraction 10* (data not shown) also a cross-peak of HMGA coupled to the C-6 position of a glucosyl unit of SDG was identified. However, due to overlapping peaks, it was not possible to determine if other groups, e.g. FeA, were attached to the same glucosyl moiety. Combining the NMR and MS data resulted in two possible configurations of SDG + HMGA +Fe A as schematically shown below:

or


In fraction 10* a structural element was identified for FeAG (see spectral data in section 4). A cross-peak between $\mathrm{C}-9$ and $\mathrm{CH}_{2}$ of EtOH led to the identification of $\mathrm{FeAG}+\mathrm{EtOH}$ (see Table 2) and showed that also FeAG was ester-linked within the lignan macromolecule via its carboxylic group. In none of the fractions cross-peaks showing the coupling between FeAG and SDG were found.
In the MS-data (Table 1) of fraction $8^{*}$, a structural element annotated as SDG + HMGA+FeAG was found. This structure might show the linkage of FeAG to the lignan macromolecule. The MS/MS data showed that glucose and glucose+HMGA could be split off. However, these data were not decisive about the position of HMGA. Therefore, it remains to be determined at which position FeAG links to SDG.
It is remarkable that based on MS analysis fragments with FeAG were annotated, and that these structural elements were not found with NMR. This might be explained based on the sensitivity to ionization. The fragment identified by MS carries a HMGA moiety. The free carboxyl moiety of this fragment is already in a deprotonated, charged state or is very easily ionized. Therefore, it is likely to give a large response on MS (Cech and Enke, 2001). NMR signals are not dependent on such phenomena, so they are proportional with the amounts present in the sample. The present data suggest that the fragment SDG+HMGA+FeAG was present in such low amounts that the NMR signals of this fragment could not be distinguished from the noise.
Table 3. NMR chemical shifts of SDG+CouAG, SDG+2HMGA and SDG+FeA.

| SDG + | $\mathrm{JAGG}^{\text {t }}$ |  |  | SDG +2 | $\mathrm{MGA}^{\text {a }}$ |  |  | SDG + |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | ${ }^{1} \mathrm{H} \delta$ | mult., $J$ (Hz) ${ }^{\text {e }}$ | ${ }^{13} \mathrm{C} \delta$ |  | ${ }^{1} \mathrm{H} \delta$ | mult, $J$ (Hz) ${ }^{\text {e }}$ | ${ }^{13} \mathrm{C} \delta$ |  | ${ }^{1} \mathrm{H} \delta$ | mult, $J$ (Hz) ${ }^{\text {e }}$ | ${ }^{13} \mathrm{C} \delta$ |
| SECO |  |  |  | SECOa |  |  |  | SECOb |  |  |  |
| 1 | - | - | 133.812 | 1 |  | - | 133.81 | 1 | - | - | 133.81 |
| 2 | 6.58 |  | 113.593 | 2 | 6.580 |  | 113.37 | 2 | 6.486 |  | 113.37 |
| 3 | - | - | 148.813 | 3 | - | - | 148.59 | 3 | - | - | 148.59 |
| 4 |  |  | 145.117 | 4 |  | - | 145.11 | 4 |  |  | 145.11 |
| 5 | 6.63 | (d) 7.75 | 115.767 | 5 | 6.616 |  | 115.76 | 5 | 6.604 |  | 115.76 |
| 6 | 6.54 | (d) 7.29 | 122.941 | 6 | 6.474 |  | 122.72 | 6 | 6.474 |  | 122.72 |
| 7a | 2.599 |  | 35.543 | 7 a | 2.616 |  | 35.12 | 7 a | 2.629 |  | 36.19 |
| 7 b | 2.599 |  |  | 7 b | 2.569 |  |  | 7 b | 2.522 |  |  |
| 8 | 2.115 |  | 41.196 | 8 | 2.169 |  | 41.19 | 8 | 1.955 |  | 41.63 |
| 9 a | 3.983 | (dd) $8.55,5.02$ | 71.198 | 9a | 3.981 |  | 70.98 | 9a | 3.957 |  | 71.41 |
| 9 b | 3.403 |  |  | 9 ab | 3.475 |  |  | 9 b | 3.381 |  |  |
| $\mathrm{OCH}_{3}$ | 3.734 |  | 56.415 | $\mathrm{OCH}_{3}$ | 3.700 |  | 56.19 | $\mathrm{OCH}_{3}$ | 3.700 |  | 56.19 |
| $1^{\prime}$ | - | - | 133.812 | Glc |  |  |  | ${ }^{1}$ | - | - | 133.81 |
| $2^{\prime}$ | 6.58 |  | 113.593 | $1{ }^{1}$ | 4.245 | (d) 7.78 | 104.46 | $2^{2}$ | 6.486 |  | 113.37 |
| ${ }^{\prime}$ | - | - | 148.813 | 2 | 3.252 |  | 75.33 | ${ }^{3}$ |  | - | 148.59 |
| 4 | - |  | 145.117 | 3 | 3.322 |  | 77.94 | 4 | - |  | 145.11 |
| $5^{\prime}$ | 6.63 | (d) 7.75 | 115.767 | 4 | 3.322 |  | 71.85 | $5 '$ | 6.604 |  | 115.76 |
| $6^{\prime}$ | 6.54 | (d) 7.29 | 122.941 | $5^{\prime}$ | 3.369 |  | 75.33 | $6^{6}$ | 6.474 |  | 122.72 |
| $7 \mathrm{a}^{\prime}$ | 2.599 |  | 35.543 | 6'a | 4.416 |  | 64.89 | 7'a | 2.628 |  | 35.76 |
| $7{ }^{\prime}$ | 2.599 |  |  | $6{ }^{\text {'b }}$ | 4.241 |  |  | $7{ }^{\text {b }}$ | 2.499 |  |  |
| $8^{\prime}$ | 2.115 |  | 41.413 |  |  |  |  | $8^{8}$ | 1.955 |  | 41.63 |
| $9 a^{\prime}$ | 4.027 | (dd) 9.74, 5.69 | 70.981 | HMGA |  |  |  | ${ }^{\text {9'a }}$ | 4.087 |  | 71.20 |
| $9 \mathrm{~b}^{\prime}$ | 3.499 |  |  |  |  | - | 172.51 | 9 b | 3.428 |  |  |
| $\mathrm{OCH}_{3}$ | 3.734 |  | 56.415 | $\begin{aligned} & \beta \mathrm{a} \\ & \beta b \end{aligned}$ | $\begin{aligned} & 2.65 \\ & 2.65 \end{aligned}$ |  | 46.19 | $\mathrm{OCH}_{3}$ | 3.700 |  | 56.19 |
| Glc |  |  |  |  |  | - | 70.76 | Glc |  |  |  |
| ${ }^{1 \prime}$ | 4.27 | (d) 7.71 | 104.679 | סa | 2.64 |  | 46.19 | ${ }^{1 \prime}$ | 4.504 | (d) 8.01 | 102.94 |
| $2{ }^{\prime \prime}$ | 3.28 |  | 78.155 | ¢b | 2.64 |  |  | $2^{\prime \prime}$ | 4.912 |  | 75.33 |
| $3 "$ | 3.4-3.15 |  | ${ }_{78.155}$ | $\varepsilon$ |  | - | 174.90 | 3" | 3.653 |  | 76.2 |
| $4{ }^{\prime \prime}$ | 3.381 |  | 72.068 | $\omega$ | 1.358 |  | 27.93 | $4{ }^{\prime \prime}$ | 3.465 |  | 71.85 |
| $5{ }^{\prime \prime}$ | 3.527 |  | 75.329 |  |  |  |  | 5" | 3.371 |  | 78.15 |
| 6"a | 4.493 | (dd) 11.92, 1.92 | 64.893 |  |  |  |  | ${ }^{6 \prime \prime}{ }^{\text {a }}$ | 3.907 |  |  |
| $6{ }^{\prime \prime}$ | 4.391 | (dd) 11.89, 6.14 |  |  |  |  |  | $6^{\prime \prime} \mathrm{b}$ | 3.759 |  | 62.93 |


| SDG + CouAG ${ }^{\text {H }}$ |  |  |  | SDG + 2 $\mathrm{HMGA}^{\text {\# }}$ |  |  | SDG + FeA ${ }^{\text {\# }}$ |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | ${ }^{1} \mathrm{H} \delta$ | mult., J(Hz) ${ }^{\text {e }}$ | ${ }^{13} \mathrm{C} \delta$ | ${ }^{1} \mathrm{H} \delta$ | mult, $J$ (Hz) ${ }^{\text {® }}$ | ${ }^{13} \mathrm{C} \delta$ |  | ${ }^{1} \mathrm{H} \delta$ | mult., $J$ (Hz) ${ }^{\text {® }}$ | ${ }^{13} \mathrm{C} \delta$ |
| Glc |  |  |  |  |  |  | Glc |  |  |  |
| $1{ }^{\prime \prime}$ | 4.17 | (d) 7.71 | 104.896 |  |  |  | $1{ }^{\prime \prime}$ | 4.174 | (d) 7.68 | 104.89 |
| $2{ }^{\prime \prime}$ | 3.21 |  | 75.329 |  |  |  | $2{ }^{\prime \prime}$ | 3.230 |  | 75.33 |
| 3"' | 3.4-3.15 |  | 78.155 |  |  |  | 3 '' | 3.369 |  | 78.15 |
| 4"' | 3.31 |  | 71.633 |  |  |  | 4"' | 3.416 |  | 71.63 |
| 5"' | 3.4-3.15 |  | 78.155 |  |  |  | $5{ }^{\prime \prime}$ | 3.218 |  | 77.72 |
| 6 "'a | 3.84 |  | 62.937 |  |  |  | 6"'a | 3.830 |  | 62.72 |
| $6 \mathrm{C} \mathrm{\prime}$ b | 3.687 |  |  |  |  |  | 6 "'b | 3.700 |  |  |
| CouA |  |  |  |  |  |  | FeA |  |  |  |
| 1* | - | - | 129.898 |  |  |  | 1* | - | - | 127.66 |
| 2* | 7.46 | (d) 8.81 | 130.985 |  |  |  | 2* | 6.94 | (d) 1.51 | 111.77 |
| 3* | 7.09 | (d) 8.83 | 117.941 |  |  |  | 3* | - |  | 149.22 |
| 4* | - | - | 160.77 |  |  |  | 4* | - | - | 150.46 |
| 5* | 7.09 | (d) 8.83 | 117.941 |  |  |  | 5* | 6.76 | (d) 8.05 | $122.93$ |
| 6* | 7.46 | (d) 8.81 | 130.985 |  |  |  | 6* | 6.91 | (dd) 8.20, 1.89 | 123.98 |
| 7* | 7.61 | (d) 16.02 | 146.204 |  |  |  | 7* | 7.52 | (d) 15.84 | 147.28 |
| 8* | 6.37 | (d) 15.58 | 116.854 |  |  |  | 8* | 6.3 | (d) 15.97 | 116.44 |
| 9* |  |  | 168.597 |  |  |  | 9* | - | - | 168.47 |
|  |  |  |  |  |  |  | $\mathrm{OCH}_{3}$ | 3.8 |  | 56.411 |
| Glc |  |  |  |  |  |  |  |  |  |  |
| 1 "" | 4.98 | (d) 7.05 | 101.853 |  |  |  |  |  |  |  |
| 2"' | 3.517 |  | 75.112 |  |  |  |  |  |  |  |
| 3"' | 3.4-3.15 |  | 77.938 |  |  |  |  |  |  |  |
| 4"' | 3.4-3.15 |  | 71.416 |  |  |  |  |  |  |  |
| $5 " 1$ | 3.235 |  | 77.938 |  |  |  |  |  |  |  |
| 6a"' | 3.91 |  | 62.502 |  |  |  |  |  |  |  |
| 6b"' | 3.71 |  |  |  |  |  |  |  |  |  |

\#Annotations are given in Table 2.
${ }^{\text {@ }}$ Due to overlapping signals, not all $J$-values could be determined.

## Discussion

The aim of this study was to determine how CouAG and FeAG are linked within the lignan macromolecule. Several structural elements of the lignan macromolecule from flaxseed hulls were identified. CouAG is attached directly via its carboxylic group to the C-6 of a glucosyl moiety of SDG. FeA is found to be linked with its carboxylic group to the C-2 position of a glucosyl moiety of SDG. These fragments show that the hydroxycinnamic acid (glucosides) are linked directly to SDG and not via the linker HMGA.
During biosynthesis, the attachment of HMGA to SDG is mediated by coenzyme A (CoA)activated HMGA (Ford et al., 2001). It was recently shown that also HDG could be a target of HMGA coupling (Struijs et al., 2007). Since linkage of HMGA to SDG and HDG occurred on the glucosyl moiety, it was expected that HMGA could also link to other molecules carrying a glucosyl moiety. The present study shows that hydroxycinnamic acids are not linked within the lignan macromolecule via HMGA. Based on the finding that hydroxycinnamic acid (glucosides) are linked directly to SDG, it is suggested that the CoAactivated hydroxycinnamic acids, identified as intermediates of the monolignol biosynthesis (Boerjan et al., 2003), are the driving force behind the linkage of the hydroxycinnamic acids to SDG.
The new structural elements SDG+CouAG and SDG+FeA are linked within the lignan macromolecule. Structures in which HMGA is linked to these elements were annotated based on MS analysis, providing indications on how they are integrated in the lignan macromolecule. These (tentative) structural elements are shown in Table 4.

Table 4. Overview of (tentative) structural elements of the lignan macromolecule from flaxseed hulls. Parts of the structures in grey are tentative. $\mathrm{n}=$ unidentified but most likely represents linkage to $\mathrm{C}-2$ or C-6 position of the glucosyl moiety.

| Element | Structure |
| :--- | :--- |
| 1 | HMGA-6-glc-SECO-glc |
| 2 | HMGA-6-glc-SECO-glc-6-HMGA |
| 3 | glc-SECO-glc-6-CouA-glc |
| 4 | HMGA-6-glc-SECO-glc-6-CouA-glc |
| 5 | glc-SECO-glc-n-FeA-glc |
| 6 | HMGA-6-glc-SECO-glc-n-FeA-glc |
| 7 | glc-SECO-glc-2-FeA |
| 8 | HMGA-6-glc-SECO-glc-2-FeA |
|  | glc-SECO-glc(-2-FeA)-6-HMGA |

Chain elongation of structural elements of SDG+CouAG or SDG+FeAG (Table 4, elements 3 and 5) might in theory occur via linkage of HMGA to the glucosyl moieties of FeAG or CouAG, or via linkage of HMGA to a glucosyl moiety of SDG. By NMR, no cross-peaks between the glucosyl moieties of CouAG or FeAG and HMGA (C- $\alpha$ or $\mathrm{C}-\varepsilon$ ) were observed. Furthermore, no fragments of SDG+2HMGA+(CouAG or FeAG) with HMGA linked to both ends of the fragment, could be annotated. These observations point to HMGA being
linked to a glucosyl moiety of SDG, and CouAG and FeAG being the terminal units of the lignan macromolecule (Table 4, elements 4 and 6). Chain elongation of SDG+FeA (Table 4, element 7) is only possible via linkage of HMGA to one of the glucosyl moieties of SDG (see also results section and Table 4, elements 8 ).
FeA is linked to the C-2 position of a glucosyl moiety of SDG. However, it is less clear how FeAG is linked with the lignan macromolecule. FeAG is found to be linked within the lignan macromolecule via its carboxylic group. There are two possible positions at which FeAG can be attached to SDG: similar to FeA to the C-2 position of a glucosyl moiety or similar to CouAG to the C-6 position (Table 4, element 5). Various examples of linkage types of hydroxycinnamic acids, but not of hydroxycinnamic acid glucosides, to sugar moieties have been described. In arabinan, FeA links to the C-2 position of arabinose (Colquhoun et al., 1994; Ishii and Tobita, 1993), whereas for anthocyanidins attachment of FeA and CouA to the C-6 position of the glucosyl moiety was shown (Fossen et al., 2005; Matsufuji et al., 2003). So also based on literature, no indications can be found about the position of attachment of FeAG to SDG.

In conclusion, CouAG and $\operatorname{FeA}(\mathrm{G})$ link within the lignan macromolecule from flaxseed hulls via ester-linkage of their carboxylic groups to glucosyl moieties of SDG. Attachment of HMGA to the glucosyl moiety of CouAG or FeAG is not observed. Therefore, it is suggested that CouAG and FeAG are terminal units of the lignan macromolecule.

Chapter 3

## Experimental

## Lignan extraction from flaxseed hulls

Lignan macromolecule was extracted from flaxseed hulls, kindly provided by Frutarom Netherlands B.V. (Veenendaal, The Netherlands). The extraction procedure was as described in Struijs et al. (2007). In short, flaxseed hulls were defatted by soxhlet extraction. The lignan macromolecule was extracted from the defatted hulls by a three-step sequential extraction with $63 \%$ (v/v) aq. EtOH for 4 hours at room temperature under continuous stirring. The extracts and the hulls were separated by filtration. The three extracts were combined and the EtOH was evaporated. The concentrated extract was lyophilized resulting in the lignan macromolecule extract.

## Saponification of the lignan macromolecule

Lignan macromolecule extract of $2 \mathrm{mg} / \mathrm{ml}$ in $63 \%$ (v/v) aq. EtOH was partially saponified with 2 mM NaOH to obtain lignan macromolecular fragments. Saponification was performed at room temperature while stirring. A reaction volume of 2.5 l was used. After 24 $h$, the reaction was stopped by lowering the pH to 6.5-7.0 with glacial HOAc.

## Sample clean up of saponified lignan macromolecule

Low molecular weight polar material was removed from the partially saponified samples by solid phase extraction (SPE; SepPak Vac, $20 \mathrm{cc} / 5 \mathrm{~g}$, C18 cartridge, Waters) following the procedure previously described in Struijs et al. (2007).

Analytical reversed phase HPLC coupled on-line to mass spectrometry (RP-HPLC/MS)
Samples, which were collected after (semi)-preparative RP-HPLC (see below), were analyzed on an analytical X-Terra C18 MS column (Waters; $3.5 \mu \mathrm{~m}$ particle size, $4.6 \times 150$ mm ) following the procedure described previously (Struijs et al., 2007).
The molecular masses and MS/MS-fragmentation patterns of the lignans and fragments of the lignan macromolecule were determined on a Thermo Finnigan LCQ Classic equipped with a ESI probe in the negative mode coupled on-line to the analytical RP-HPLC.

## Purification by (semi)-preparative RP-HPLC

Fragments of the lignan macromolecule obtained by partial saponification were purified by (semi)-preparative RP-HPLC. For purification of $100-600 \mathrm{mg}$ fragments of the lignan macromolecule, an X-Terra C18 MS column of $50 \times 100 \mathrm{~mm}$ (Waters; $5 \mu \mathrm{~m}$ particle size, OBD) with an X-Terra C18 MS guard column (Waters; $5 \mu \mathrm{~m}$ particle size, $19 \times 10 \mathrm{~mm}$ ) was used. For purification of 100 mg fragments of the lignan macromolecule or less, the samples were separated on a semi-preparative X-Terra C18 MS column (Waters; $5 \mu \mathrm{~m}$ particle size, $29 \times 150 \mathrm{~mm}$, OBD) with an X-Terra C18 MS guard column (Waters; $5 \mu \mathrm{~m}$ particle size, $19 \times 10 \mathrm{~mm}$ ). The separation protocols used were similar as described previously (Struijs et al., 2007).

## Nuclear magnetic resonance (NMR)

NMR spectra were recorded on a Bruker AMX-500 spectrometer located at the Wageningen NMR Centre as described previously (Struijs et al., 2007). ${ }^{1} \mathrm{H}$ and ${ }^{13} \mathrm{C}$ proton decoupled spectra were recorded. All 2D COSY spectra were acquired using the double quantum filtered (DQF) method with a standard pulse sequence delivered by Bruker. For the 2D HMBC spectrum a standard gradient enhanced 2D HMQC pulse sequence delivered by Bruker, was changed into a HMBC sequence.

## Spectral data

trans-CouAG+EtOH (fraction 9*): ${ }^{1} \mathrm{H}$ NMR ( $500.13 \mathrm{MHz}, \mathrm{CD}_{3} \mathrm{OD}$ ) $\delta 7.54(2 \mathrm{H}, d, J=8.74$ Hz, H-2/6), 7.11 (2 H, d, J=8.74 Hz, H-3/5), 7.62 ( $1 \mathrm{H}, d, J=15.95 \mathrm{~Hz}, \mathrm{H}-7$ ), 6.39 ( $1 \mathrm{H}, d$, $J=16.05 \mathrm{~Hz}, \mathrm{H}-8$ ), 4.97 ( $1 \mathrm{H}, d, J=7.28 \mathrm{~Hz}, \mathrm{H}-1^{\prime}$ ), $3.5-3.3$ ( $4 \mathrm{H}, m, \mathrm{H}-2^{\prime} / 3^{\prime} / 5^{\prime} / 4^{\prime}$ ), 3.90 ( 1 H, dd, J=12.13, $2.02 \mathrm{~Hz}, 1 \mathrm{H}, \mathrm{H}-6 \mathrm{a}$ ), 3.70 ( $1 \mathrm{H}, d d, J=12.09,5.53 \mathrm{~Hz}, 1 \mathrm{H}, \mathrm{H} 6 \mathrm{~b}$ ), 4.23 (2 $\left.\mathrm{H}, q, J=7.14 \mathrm{~Hz}, \mathrm{CH}_{2}\right), 1.31\left(3 \mathrm{H}, t, J=7.17 \mathrm{~Hz}, \mathrm{CH}_{3}\right)$.
trans-FeAG+EtOH (fraction $10 *$ ): ${ }^{1} \mathrm{H}$ NMR ( $500 \mathrm{MHz}, \mathrm{CD}_{3} \mathrm{OD}$ ) $\delta 7.62(1 \mathrm{H}, d, J=15.93$ Hz, H-7), 7.24 ( $1 \mathrm{H}, d, J=1.24 \mathrm{~Hz}, \mathrm{H}-2$ ), 7.15 ( $1 \mathrm{H}, d d, J=8.47,1.53 \mathrm{~Hz}, \mathrm{H}-6$ ), 7.17 ( $1 \mathrm{H}, d$, $J=8.45 \mathrm{~Hz}, \mathrm{H}-5), 6.43$ ( $1 \mathrm{H}, d, J=16.00 \mathrm{~Hz}, \mathrm{H}-8$ ), 4.97 ( $1 \mathrm{H}, d, J=7.38 \mathrm{~Hz}, \mathrm{H}-1$ '), 3.89 ( 3 H , s, CH3 ), 3.89 ( $1 \mathrm{H}, d d, J=11.90,1.89 \mathrm{~Hz}, \mathrm{H}-6 \mathrm{a}$ '), 3.69 ( $1 \mathrm{H}, d d, J=11.90,5.31 \mathrm{~Hz}, \mathrm{H} 6 \mathrm{~b}$ ) , 3.51 ( $2 \mathrm{H}, \mathrm{H}-2^{\prime} / 3^{\prime}$ ), $3.40\left(1 \mathrm{H}, \mathrm{H}-4^{\prime}\right)$, 3.45 ( $1 \mathrm{H}, \mathrm{H}-5^{\prime}$ ), $4.23\left(2 \mathrm{H}, q, J=7.1 \mathrm{~Hz}, \mathrm{CH}_{2}\right.$ ), 1.31 $\left(3 \mathrm{H}, t, J=7.1 \mathrm{~Hz}, \mathrm{CH}_{3}\right)$.
${ }^{13} \mathrm{C}$ NMR (125.77 MHz, CD ${ }_{3} \mathrm{OD}$ ) $\delta 130.6$ (C-1), 112.5 (C-2), 151.0 (C-3), 150.1 (C-4), 117.4 (C-5), 123.5 (C-6) 145.9 (C-7), 117.5 (C-8), 169.0 (COOD), $56.8\left(\mathrm{OCH}_{3}\right), 102.2$ (C1’), 74.9 (C-2’), 78.3 (C-3’), 71.3 (C-4’), 77.9 (C-5'), 62.5 (C-6’), $61.6\left(\mathrm{CH}_{2}\right), 14.69\left(\mathrm{CH}_{3}\right)$.

## References

Bakke, J.E., Klosterman, H.J., 1956. A new diglucoside from flaxseed. Proc. North Dakota Acad. Sci. 10, 18-21.

Boerjan, W., Ralph, J., Baucher, M., 2003. Lignin biosynthesis. Annu. Rev. Plant Biol. 54, 519-546.

Cech, N.B., Enke, C.G., 2001. Practical implications of some recent studies in electrospray ionization fundamentals. Mass Spectrom. Rev. 20, 362-387.

Colquhoun, I.J., Ralet, M.C., Thibault, J.F., Faulds, C.B., Williamson, G., 1994. Structure identification of feruloylated oligosaccharides from sugar-beet pulp by NMR-spectroscopy. Carbohydr. Res. 263, 243-256.

Dabrowski, K.J., Sosulski, F.W., 1984. Composition of free and hydrolyzable phenolic acids in defatted flours of ten oilseeds. J. Agric. Food Chem. 32, 128-130.

Eliasson, C., Kamal-Eldin, A., Andersson, R., Aman, P., 2003. High-performance liquid chromatographic analysis of secoisolariciresinol diglucoside and hydroxycinnamic acid glucosides in flaxseed by alkaline extraction. J. Chromatogr. A 1012, 151-159.

Ford, J.D., Huang, K.S., Wang, H.B., Davin, L.B., Lewis, N.G., 2001. Biosynthetic pathway to the cancer chemopreventive secoisolariciresinol diglucoside-hydroxymethyl glutaryl ester- linked lignan oligomers in flax (Linum usitatissimum) seed. J. Nat. Prod. 64, 1388-1397.

Fossen, T., Rayyan, S., Holmberg, M.H., Nateland, H.S., Andersen, O.M., 2005. Acylated anthocyanins from leaves of Oxalis triangularis. Phytochemistry 66, 1133-1140.

Ishii, T., Tobita, T., 1993. Structural characterization of feruloyl oligosaccharides from spinach-leaf cell-walls. Carbohydr. Res. 248, 179-190.

Johnsson, P., Peerlkamp, N., Kamal-Eldin, A., Andersson, R.E., Andersson, R., Lundgren, L.N., Aman, P., 2002. Polymeric fractions containing phenol glucosides in flaxseed. Food Chem. 76, 207-212.

Kamal-Eldin, A., Peerlkamp, N., Johnsson, P., Andersson, R., Andersson, R.E., Lundgren, L.N., Aman, P., 2001. An oligomer from flaxseed composed of secoisolariciresinoldiglucoside and 3-hydroxy-3-methyl glutaric acid residues.
Phytochemistry 58, 587-590.
Klosterman, H.J., Smith, F., Clagett, C.O., 1955. The constitution of linocinnamarin. J. Am. Chem. Soc. 77, 420-421.

Liggins, J., Grimwood, R., Bingham, S.A., 2000. Extraction and quantification of lignan phytoestrogens in food and human samples. Anal. Biochem. 287, 102-109.

Matsufuji, H., Otsuki, T., Takeda, T., Chino, M., Takeda, M., 2003. Identification of reaction products of acylated anthocyanins from red radish with peroxyl radicals. J. Agric. Food Chem. 51, 3157-3161.

Meagher, L.P., Beecher, G.R., Flanagan, V.P., Li, B.W., 1999. Isolation and characterization of the lignans, isolariciresinol and pinoresinol, in flaxseed meal. J. Agric. Food Chem. 47, 3173-3180.

Milder, I.E.J., Arts, I.C.W., van de Putte, B., Venema, D.P., Hollman, P.C.H., 2005. Lignan contents of Dutch plant foods: a database including lariciresinol, pinoresinol, secoisolariciresinol and matairesinol. Br. J. Nutr. 93, 393-402.

Qiu, S.-X., Lu, Z.-Z., Luyengi, L., Lee, S.K., Pezzuto, J.M., Farnsworth, N.R., Thompson, L.U., Fong, H.H.S., 1999. Isolation and characterization of flaxseed (Linum usitatissimum) constituents. Pharm. Biol. 37, 1-7.

Sicilia, T., Niemeyer, H.B., Honig, D.M., Metzler, M., 2003. Identification and stereochemical characterization of lignans in flaxseed and pumpkin seeds. J. Agric. Food Chem. 51, 1181-1188.

Struijs, K., Vincken, J.-P., Verhoef, R., van Oostveen-van Casteren, W.H.M., Voragen, A.G.J., Gruppen, H., 2007. The flavonoid herbacetin diglucoside as a constituent of the lignan macromolecule from flaxseed hulls. Phytochemistry 68, 1227-1235.

Thompson, L.U., Seidl, M.M., Rickard, S.E., Orcheson, L.J., Fong, H.H.S., 1996.
Antitumorigenic effect of a mammalian lignan precursor from flaxseed. Nutr. Cancer 26, 159-165.

Vanharanta, M., Voutilainen, S., Lakka, T.A., van der Lee, M., Adlercreutz, H., Salonen, J.T., 1999. Risk of acute coronary events according to serum concentrations of enterolactone: a prospective population-based case-control study. Lancet 354, 2112-2115.

Ward, W.E., Yuan, Y.V., Cheung, A.M., Thompson, L.U., 2001. Exposure to purified lignan from flaxseed (Linum usitatissimum) alters bone development in female rats. Br. J. Nutr. 86, 499-505.

Westcott, N.D., Muir, A.D., 1996. Process for extracting and purifying lignans and cinnamic acid derivatives from flaxseed. International Patent WO 9630468.

## Chapter 4 <br> The chain length of the lignan macromolecule from flaxseed hulls is determined by the incorporation of hydroxycinnamic acid glucosides

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#### Abstract

The lignan macromolecule from flaxseed hulls is composed of secoisolariciresinol diglucoside (SDG) and herbacetin diglucoside (HDG) moieties ester-linked by 3-hydroxy-3-methyl-glutaric acid (HMGA), and of p-coumaric acid glucoside (CouAG) and ferulic acid glucoside (FeAG) moieties ester-linked directly to SDG. The linker molecule HMGA was found to account for $11 \%(\mathrm{w} / \mathrm{w})$ of the lignan macromolecule. The molar extinction coefficients of SDG, HDG, CouAG and FeAG, were determined to be 5838, 10347, 19474, and $12133 \mathrm{M}^{-1} \mathrm{~cm}^{-1}$, respectively. Based on these values and RP-HPLC data, it was determined that SDG contributes for $56 \mathrm{~mol} \%$ to the phenolic constituents, while CouAG, FeAG, and HDG contribute for 23, 15 and $6 \mathrm{~mol} \%$, respectively. Analysis of fractions of the lignan macromolecule showed that the higher the molecular mass, the higher the proportion of SDG was. An inverse relation between the molecular mass and the proportion (\%) hydroxycinnamic acid glucosides (CouAG + FeAG) was found. Together with the structural information of oligomers of the lignan macromolecule obtained after partial saponification, it is hypothesized that the amount of hydroxycinnamic acid glucosides present during biosynthesis determines the chain length of the lignan macromolecule. Furthermore, the chain length was estimated from a model describing the lignan macromolecule based on structural and compositional data. The average chain length of the lignan macromolceule was calculated to be three SDG moieties with two hydroxycinnamic acid glucosides at the terminal positions, with a variation between one and seven SDG moieties.


## Introduction

Lignans are phenolic compounds, which, by definition, are composed of two propylbenzene moieties, which are linked by a bond between the 8 and the 8 - position (Moss, 2000). These propyl-benzene structures, mostly coniferyl alcohol, are biosynthesized through the phenylpropanoid pathway and stereospecifically dimerized into the lignan pinoresinol (PINO) (Davin et al., 1997). Subsequently, reduction, oxidation, dehydrogenation and addition reactions lead to the formation of a broad range of lignans, which are present in both aglyconic and in glycosylated forms (Ford et al., 2001; Hano et al., 2006; von Heimendahl et al., 2005; Youn et al., 2005).
Secoisolariciresinol diglucoside (SDG) is the most abundant lignan present in flaxseed (Johnsson et al., 2000). It is synthesized from PINO by the action of pinoresinollariciresinol reductase (PLR) (von Heimendahl et al., 2005), followed by glucosylation by an UDPG:glucosyltransferase (Ford et al., 2001). The formation of SDG takes place in the outer layer of the seed (Hano et al., 2006). Therefore, the concentration of SDG found in flaxseed hulls is higher than that of whole seeds (Madhusudhan et al., 2000). The lignans in flaxseeds (hulls) are parts of an oligomeric structure, called the lignan macromolecule. Within this lignan macromolecule, SDG is ester-linked by 3-hydroxy-3-methyl-glutaric acid (HMGA) (Kamal-Eldin et al., 2001; Klosterman and Smith, 1954). Also herbacetin diglucoside (HDG), ferulic acid glucoside (FeAG) and p-coumaric acid glucoside (CouAG) are part of the lignan macromolecule (Johnsson et al., 2002; Struijs et al., 2007).
Analysis of seeds at different developmental stages showed that hardly any free SDG is present in developing seeds. Almost directly after its formation, SDG is incorporated within the lignan macromolecule (Ford et al., 2001; Hano et al., 2006). Oligomers of SDG and HMGA are formed by ester-linkage with CoA-activated HMGA (Ford et al., 2001). Molecules consisting of one or two SDG moieties with one, two or three HMGA moieties have been identified (Ford et al., 2001). Just like SDG, CouAG and FeAG are incorporated in an alkali labile structure in early stages of seed development (Ford et al., 2001; Hano et al., 2006).
Only limited data about the composition and size of the lignan macromolecule are available. In 2001, a model of the lignan macromolecule consisting of 5 SDG units being esterified with 4 HMGA residues was published (Kamal-Eldin et al., 2001). However, in later studies, variation in the proportions of constituents was observed between fractions of the lignan macromolecule separated by reversed-phase solid-phase extraction (Johnsson et al., 2002; Strandas et al., 2008), indicating that the lignan macromolecule represents an array of molecules with varying composition. In addition, it has been shown that the flavonoid HDG links, just like SDG, within the lignan macromolecule via HMGA (Struijs et al., 2007), while the hydroxycinnamic acid glucosides (CouAG and FeAG) are linked directly to the glucosyl moieties of SDG through their carboxylic groups (Struijs et al., 2008).
More precise data about the composition and size of the lignan macromolecule could give direction to further research on the biosynthesis of the lignan macromolecule. The aim of present research is to investigate the composition and size of the lignan macromolecule from flaxseed hulls in order to be able to determine the correlation between the composition and the size of the molecules of the lignan macromolecule.

## Results and discussion

## Quantification of the constituents of the lignan macromolecule

The components described to be part of the lignan macromolecule from flaxseed hulls, can be divided into two groups: the phenolic constituents (see later) and the linker molecule HMGA. Fig. 1 shows the GC profile of HMGA from fully saponified lignan macromolecule. Peak 2 was identified as HMGA by comparing the profiles with those of the blanks and the authentic standard. To confirm its identity, peak 4 was analyzed by GCMS. In the insert, the MS spectrum of peak $2(\mathrm{RT}=10.9 \mathrm{~min})$ is shown. By comparing the spectrum with the spectrum of an authentic standard of HMGA and with literature values (Ford et al., 2001), this peak was identified as HMGA. The concentration HMGA was calculated as $111 \pm 13 \mu \mathrm{~g} / \mathrm{mg}$ macromolecule. This number corresponds with the amount determined on the basis of the recovered weights of HMGA isolated from the lignan macromolecule as reported previously (Prasad, 2004).


Figure 1. GC profile of fully saponified lignan macromolecule after derivatisation. $1=$ pyridine, $2=$ derivatizing agent, $3=$ glutaric acid (internal standard), $4=$ HMGA. In the insert the GC-MS spectrum of peak 4. $m / z$-ratios $[\mathrm{M}+\mathrm{H}]^{+}$marked gray are also present in other fractions eluting at different retention times and are, therefore, not originating from HMGA.

Besides the $11.1 \%$ ( $\mathrm{w} / \mathrm{w}$ ) of HMGA, the lignan macromolecule consisted of phenolic compounds, mainly SDG, CouAG, FeAG and HDG. To be able to determine the proportion of each of these phenolic constituents, their molar extinction coefficients were determined, as these data have not been reported in literature.
The molar extinction coefficients at 280 nm of purified SDG, CouAG, FeAG and HDG (Struijs et al., 2007) are given in Table 1. In this table, also the purity of the compounds,

## Chain length is determined by the incorporation of hydroxycinnamic acid glucosides

based on NMR signals, is given. The purity of the compounds was found to be close to $90 \%$ or higher.

Table 1. Molar extinction coefficients of the four main phenolic constituents of the lignan macromolecule.

| Constituent | Purity | Molar extinction coefficient at <br> $280 \mathrm{~nm}\left(\mathrm{M}^{-1} \mathrm{~cm}^{-1}\right)$ | SD <br> absolute (\%) | $\lambda_{\max }(\mathrm{nm})$ |
| :---: | :---: | :---: | :---: | :---: |
| CouAG | $92 \%$ | 19474 | $1043(5.4 \%)$ | 297 |
| FeAG | $98 \%$ | 12133 | $292(2.4 \%)$ | $291+316$ |
| HDG | $89 \%$ | 10347 | $158(1.5 \%)$ | 271 |
| SDG | $100 \%$ | 5838 | $201(3.4 \%)$ | 281 |

In Table 2, the composition of the lignan macromolecule from flaxseed hulls is given. On a molar basis, SDG was found to be the most abundant constituent, followed by CouAG, FeAG, and HDG. Recalculation of the molar proportions of the phenolic compounds into weight ratios showed that the lignan macromolecule consists of $62.0 \%$ (w/w) SDG, $5.7 \%$ HDG, $12.2 \%$ CouAG and $9.0 \%$ FeAG. These values correspond with literature values where different methods were used (Prasad, 2004)' (Eliasson et al., 2003).
The proportion of CouAG was higher than the proportion of FeAG. This might be explained by the biosynthetic pathway of these compounds. During monolignol biosynthesis, CouA is formed earlier than FeA (Boerjan et al., 2003). Besides, it was found that incorporation of hydroxycinnamic acids in the lignan macromolecule occurs at early stages of seed development (Ford et al., 2001). Based on these literature data and the results obtained in the present study, it is suggested that when CouA is glucosylated and/or it is incorporated within the lignan macromolecule, it is not available anymore for conversion into FeA and FeAG. This might explain the lower proportion of FeAG in the lignan macromolecule compared to CouAG.

Table 2. Molar proportions and weight percentages of the constituents of the lignan macromolecule.

| Constituent | Molar proportions* of <br> phenolic constituent (\%) | $\pm$ SD | Weight percentage** <br> $(\%, \mathrm{w} / \mathrm{w})$ | $\pm$ SD |
| :--- | ---: | ---: | ---: | ---: |
| CouAG | 23.1 | $\pm 2.8$ | 12.2 |  |
| FeAG | 15.6 | $\pm 0.9$ | 9.0 |  |
| HDG | 5.6 | $\pm 0.4$ | 5.7 |  |
| SDG | 55.7 | $\pm 2.0$ | 62.0 |  |
| HMGA |  |  | 11.1 | $\pm 1.3$ |

*Determined based on peak area of the RP-HPLC profiles (not shown) and the molar extinction coefficient (Table 1).
** The weight percentage ( $\%, \mathrm{w} / \mathrm{w}$ ) of HMGA was determined by GC. The weight percentage (\%, $\mathrm{w} / \mathrm{w}$ ) of phenolic constituents were calculated from molar proportions.

## Molecular mass of the lignan macromolecule

Since precise data on the molecular mass of the macromolecule are lacking, MALDI-TOF MS analysis was performed (Fig. 2). The pattern of peaks showed variation in composition and size of the molecules of the lignan macromolecule. The spectrum showed four groups of peaks, each group differing $812(\mathrm{~m} / \mathrm{z} ; 812=830$-water) from each other, corresponding to a difference of SDG+HMGA. Within each group, a repetitive pattern of five clusters (AE) of peaks was observed. The differences in $m / z$-ratios between the clusters varied. An
enlargement of the first group, in which the individual peaks in the five clusters are marked, is shown in the insert of Fig. 2. Each of the five clusters showed a unique peak pattern. For example, in cluster A, two peaks were observed while in cluster E six peaks were distinguished. The differences in $m / z$-ratio between the peaks mostly corresponded to 12 , 14,16 or 18 . The difference in $m / z$-ratio of 16 was assigned as the difference between the sodium versus the potassium adduct.


Figure 2. MALDI-TOF MS spectrum of the lignan macromolecule measured in the positive reflector mode. Four groups of 5 clusters (A-E) of peaks are shown. Each group represents an elongation of the macromolecular chain by SDG+HMGA compared to the previous group. In the insert a zoomspectrum of $1^{\text {st }}$ group is depicted. Peak assignments are listed in Table 3.

In Table 3 the peaks of the first group are listed together with their molecular masses and their (tentative) assignments. Peaks were ascribed as combinations of SDG, HMGA, CouA(G), FeA(G), and HDG or other phenolic compounds described to be present in flaxseed: the lignans matairesinol (MAT) (Liggins et al., 2000), isolariciresinol (iso-LARI) (Meagher et al., 1999), pinoresinol (PINO) (Meagher et al., 1999) and lariciresinol (LARI) (Sicilia et al., 2003), the flavonoid kaempferol diglucoside (Qiu et al., 1999), and the hydroxycinnamic acids caffeic acid glucoside (Westcott and Muir, 1996) and sinapic acid (Dabrowski and Sosulski, 1984).
Peak A1, for example, was assigned as 2SDG+HMGA and peak A2 as the potassiumadduct of this same molecule. In the B-cluster, peak B3 was assigned as A1 elongated with one HMGA moiety, and in the C-cluster, peak C1 was assigned as A1 plus two HMGA moieties. Several peaks within the D and E clusters were identified as molecules composed of SDG and HMGA to which hydroxycinnamic acids or their glucosides were attached. Most other peaks were partially assigned by looking at differences in $\mathrm{m} / \mathrm{z}$-ratios between peaks and ascribing the differences to components present in flaxseeds. For example, peak

Chain length is determined by the incorporation of hydroxycinnamic acid glucosides
Table 3. Assignments of $m / z$-ratios found the first group of peaks in the MALDI-TOF MS spectrum of the lignan macromolecule as shown in Fig. 2.

| peak | $\mathrm{m} / \mathrm{z}$-ratio [M+Na] ${ }^{+}$ | possible assignment [M+Na] ${ }^{+}$ | partially assigned peaks* |
| :---: | :---: | :---: | :---: |
| A1 | 1521.6 | 2SDG+HMGA |  |
| A2 | 1537.6 | 2SDG+HMGA $[\mathrm{M}+\mathrm{K}]^{+* *}$ |  |
| B1 | 1605.6 | SDG+2HMGA+HDG | 1521.6 + HMGA |
| B2 | 1647.7 |  |  |
| B3 | 1665.7 | 2SDG+2HMGA |  |
| B4 | 1679.8 |  |  |
| B5 | 1695.7 |  |  |
| C1 | 1809.9 | 2SDG+3HMGA | 1521.6 + 2 HMGA |
|  |  |  | 1647.9 + Glc |
| C2 | 1823.8 |  | 1679.8 + HMGA |
|  |  |  | 1647.7 + FeA |
| C3 | 1837.9 |  | 1679.8 + FeA |
| C4 | 1855.9 |  |  |
| C5 | 1873.8 |  |  |
| D1 | 1971.9 |  | 1647.7 + 2Glc |
|  |  |  | 1647.7 + CafAG |
|  | 1973.9 | 2SDG+2HMGA+CouAG | 1665.7 + CouAG |
| D2 | 1985.9 | 2SDG $+3 \mathrm{HMGA}+\mathrm{FeA}$ | 1823.8 + Glc |
|  |  |  | 1809.9 + FeA |
|  |  |  | 1647.7 + FeAG |
|  | 1987.9 |  | $1971.9+16$ |
| D3 | 1999.9 |  | $1837.9+$ Glc |
|  |  |  | 1823.8 + FeA |
|  |  |  | 1855.9 + HMGA |
|  | 2001.9 | $2 \mathrm{SDG}+3 \mathrm{HMGA}+\mathrm{FeA}[\mathrm{M}+\mathrm{K}]^{+* *}$ | $1985.9+16$ |
|  | 2003.9 | $2 \mathrm{SDG}+2 \mathrm{HMGA}+\mathrm{FeAG}$ | 1665.7 + FeAG |
| D4 | 2017.9 |  | 1855.9 + Glc |
|  |  |  | 1679.8 + FeAG |
|  | 2019.9 | $2 \mathrm{SDG}+2 \mathrm{HMGA}+\mathrm{FeAG}[\mathrm{M}+\mathrm{K}]^{+* *}$ | $2003.9+16$ |
| D5 | 2031.9 |  | $1855.9+$ FeA |
|  | 2033.9 |  | $2017.9+16$ |
| E1 | 2131.0 |  | 1823.8 + CouAG |
| E2 | 2148.1 | 2SDG $+3 \mathrm{HMGA}+\mathrm{FeAG}$ | 1985.9 + Glc |
|  |  |  | 1809.9 + FeAG |
|  |  |  | 1823.8 + CafAG |
| E3 | 2164.0 (av) |  |  |
| E4 | 2184.0 (av) |  |  |
| E5 | 2199.9 (av) | 2SDG+HMGA+2FeAG | 2003.9 + FeAG |
| E6 | 2215.9 (av) | $2 \mathrm{SDG}+\mathrm{HMGA}+2 \mathrm{FeAG}[\mathrm{M}+\mathrm{K}]^{+* *}$ | $2148.1+16$ |

(av) = most dominant isotope is not the first isotope peak, assignments calculated based on average molecular weights in stead of monoisotopic molecular weights.

* Differences in $m / z$-ratios between peaks could be attributed to constituents of the lignan macromolecule.
** Mostly fragments were assigned as sodium adducts $[\mathrm{M}+\mathrm{Na}]^{+}$but in some occasions the potassium adduct $[\mathrm{M}+\mathrm{K}]^{+}$was found. The potassium adduct shows a differences of 16 with the sodium adduct.

E1 $\left(\mathrm{m} / \mathrm{z}=2131[\mathrm{M}-\mathrm{H}]^{-}\right)$was partially assigned as peak C2 $\left(\mathrm{m} / \mathrm{z}=1823.8[\mathrm{M}-\mathrm{H}]^{-}\right)$plus CouAG ( $\mathrm{m} / \mathrm{z}=325[\mathrm{M}-\mathrm{H}]^{-}$). Differences in $m / z$-ratios with unidentified peaks corresponding to HMGA, glucose, FeA(G), CafAG, or CouAG were found, suggesting that these peaks were derivatives of the lignan macromolecule.

Based on the peaks assigned and the difference in $m / z$-ratios between the groups, it was shown that the lignan macromolecule is a collection of molecules of various sizes and compositions. Molecules ranging from 2SDG +1 HMGA ( $M_{\mathrm{r}}=1.5 \mathrm{kDa}$ ) up to at least 5 SDG $+6 \mathrm{HMGA}\left(M_{\mathrm{r}}=4.3 \mathrm{kDa}\right.$ ) were identified by MALDI-TOF MS as being part of the lignan macromolecule. It still remains to be identified what the maximum molecular mass is because the MALDI-TOF MS signal was declining with increasing molecular mass.

## Subunit composition of fractions of the lignan macromolecule

To investigate the variation in composition and size in more detail, the lignan macromolecule was fractionated by GPC as indicated in Fig. 3. The total lignan macromolecule eluted as one broad peak. Reinjection of the collected fractions on the same GPC column (insert in Fig. 3) showed that fractions, which differed in molecular mass, were obtained.


Figure 3. GPC profile of the lignan macromolecule. The collected fractions are indicated. In the insert, the GPC profiles of the collected fractions are shown, reinjected on the same column.

The composition of the collected GPC-fractions was determined by analyzing the fractions on analytical RP-HPLC after full saponification (Fig. 4). In Fig. 5 the molar proportions of the four main phenolic constituents per GPC-fraction are shown. In the first five fractions, SDG was the predominant constituent, whereas in the fraction 7 and 8 CouAG and FeAG were predominating. With increasing retention times (thus a decrease molecular weight) the relative molar proportion of SDG decreased from 73 to $27 \%$, whereas the proportion of

Chain length is determined by the incorporation of hydroxycinnamic acid glucosides
CouAG increased from 12 to 52\%. FeAG also showed an increase in proportion (from 10 to $18 \%$ ), but this increase was smaller than that for CouAG.


Figure 4. RP-HPLC profiles of the saponified fractions of the lignan macromolecule collected after GPC. $1=$ CouAG, $2=$ FeAG, $3=\mathrm{HDG}, 4=$ SDG.

The increase in proportions of CouAG and FeAG provided additional information about the structure of the molecules in the lignan macromolecule. When CouAG and FeAG would have been randomly distributed over a molecule, an increase in chain length is not expected to result in a change in ratio of SDG:(CouAG + FeAG). Since an inverse relation between molecular mass and proportion of CouAG + FeAG was observed, it is suggested that the hydroxycinnamic acid glucosides were the terminating units. The molar ratio of SDG:(CouAG + FeAG) of 1:2 found in the fraction with the lowest molecular mass (fraction 8), also points in the direction of the presence of hydroxycinnamic acid glucosides on each terminal position of SDG. Also previous research showed indications for such a structure (Struijs et al., 2008).

These data suggest that chain elongation of the lignan macromolecule stops after incorporation of a hydroxycinnamic acid glucoside. It is hypothesized that the chain length of the molecules of the lignan macromolecule is controlled by the concentration free hydroxycinnamic acid glucosides present during biosynthesis. In early stages of flaxseed development, high concentrations of free CouAG and FeAG are detected, while in later stages, the concentration free hydroxycinnamic acid glucosides decreases (Hano et al., 2006). In addition, it has been observed that higher oligomers of SDG and HMGA are formed in later stages of seed development (Ford et al., 2001). These observations suggest that in early stages of seed development molecules with short chain lengths are terminated by the, in those stages, highly abundant hydroxycinnamic acid glucosides. In later stages, when the hydroxycinnamic acid glucosides are less abundant, longer chains are
biosynthesized. This is in accordance with the hypothesis that the concentration of free hydroxycinnamic acid glucosides determines the chain length of the lignan macromolecule.


Figure 5. Relative molar proportions of CouAG (black), FeAG (white), HDG (dark gray), and SDG (grey) in the fractions collected by GPC after saponification.

## Schematic representation of the lignan macromolecule

Taking all results into account, the lignan macromolecule can be defined as a collection of molecules with various molecular masses, which are composed of one or more SDG moieties linked to one or more HMGA and/or hydroxycinnamic acid glucoside residues. The lignan macromolecule is schematically represented by Fig 6A. SDG (or HDG), CouAG, FeAG (CouAG and FeAG are indicated in Fig. 6A as HCAG), and HMGA are the building blocks of the lignan macromolecule. SDG (or HDG) moieties link to each other via HMGA (Struijs et al., 2007), while CouAG and FeAG link directly to SDG (Struijs et al., 2008). Also FeA is a constituent of the lignan macromolecule (Struijs et al., 2008), but present in such low amounts that it is not accounted for.

The schematic representation should comply with the structures assigned after MALDITOF MS analysis (Table 3: 2SDG+HMGA; $n_{1}=n_{3}=0, n_{2}=1$ up to 5SDG+5HMGA; $n_{1}=$ $0, n_{2}=4, n_{3}=1$ ). Based on these MALDI-TOF MS data, it was expected that a combination of SDG and HMGA is the basic unit of the molecules of the lignan macromolecule. However, also the composition of the GPC-fractions should be taken into account. The ratio of 1:2 between SDG and CouAG (+ FeAG) showed that CouAG-SDG-CouAG is a possible member of the lignan macromolecule, which led to the suggestion that the hydroxycinnamic acid glucosides are present at the terminal positions (as discussed above). Therefore, the representation (Fig. 6A) should also reflect the possibility of CouAG and FeAG at the terminal positions.

## Chain length is determined by the incorporation of hydroxycinnamic acid glucosides

By definition, the smallest molecules being part of the lignan macromolecule are SDG+HMGA or SDG+CouAG (or + FeAG). For these molecules the following is true: $\mathrm{n}_{2}=$ 0 and $n_{1}$ or $n_{3}=1$. For larger molecules, CouAG, FeAG or HMGA can or can not be present at the terminal position so the value for $n_{1}$ and $n_{3}$ can be either 0 or 1 . The value for $n_{2}$ will vary with the chain length.
A

B


Figure 6. (A) Schematic representation of the lignan macromolecule from flaxseed hulls. $n_{1}+n_{2}+n_{3}$ $\geq 1$. HCAG = CouAG or FeAG. Modeled values for $n_{2}+1$ can be found in Table 4. (B): An example of a representative of the average lignan macromolecule is given, in which $n_{1}=n_{3}=1$ and HCAG $=$ CouAG, $\mathrm{n}_{2}=2, /=$ glycosidic bond, and $\square=$ ester bond.

## Average chain length of the lignan macromolecule

Experimentally, the chain length could not be measured and the value of $n_{2}$ remained unidentified. To be able to estimate $\mathrm{n}_{2}$, a model was developed which predicts chain length based on the compositional (current study) and structural data (Struijs et al., 2008).
A lignan macromolecular chain is described as a combination of the different building blocks:
$\mathrm{LM}=\left(\mathrm{n}_{2}+1\right)$ SDG $+\left(\mathrm{n}_{2}+\mathrm{a}\right) \mathrm{HMGA}+\mathrm{b}(\mathrm{CouAG}+\mathrm{FeAG}) \quad$ (equation 1)
in which LM $=$ the total lignan macromolecule, $\left(\mathrm{n}_{2}+1\right)=$ number of SDG units, a $=$ number of terminal units HMGA, $\mathrm{b}=$ number of terminal units hydroxycinnamic acid glucosides (CouAG + FeAG). The following constraints are active: $\mathrm{n}_{2} \geq 0, \mathrm{a} \geq 0, \mathrm{~b} \geq 0, \mathrm{a}+\mathrm{b} \leq 2$. In equation 1 , $a+b$ are the total terminal units of HMGA plus the hydroxycinnamic acid glucosides. In Fig. 6A, they are represented by $n_{1}+n_{3}$.
Assuming that the molar proportion of SDG ( $\mathrm{y}_{\mathrm{SDG}}$ ), the molar proportion of hydroxycinnamic acid glucosides ( $\mathrm{y}_{(\mathrm{CouAG}+\mathrm{FeAG})}$ ), and the molar proportion of HMGA (унмGA) are forming the total lignan macromolecule, the following is true:

```
\(\mathrm{y}_{\mathrm{SDG}}+\mathrm{y}_{\mathrm{HMGA}}+\mathrm{y}_{(\mathrm{CouAG}+\mathrm{FeAG})}=1\)
in which
\(y_{\text {SDG }}=\left(\mathrm{n}_{2}+1\right) /\left(\left(\mathrm{n}_{2}+1\right)+\left(\mathrm{n}_{2}+\mathrm{a}\right)+\mathrm{b}\right)\)
\(y_{\text {HMGA }}=a /\left(\left(n_{2}+1\right)+\left(n_{2}+a\right)+b\right)\)
\(\mathrm{y}_{\text {(CouAG }+ \text { FeAG })}=\mathrm{b} /\left(\left(\mathrm{n}_{2}+1\right)+\left(\mathrm{n}_{2}+\mathrm{a}\right)+\mathrm{b}\right)\)
```

(equations 2)

The following equations describe the molar proportions of SDG, HMGA and hydroxycinnamic acid glucosides in the lignan macromolecule as a set of linear equations.

```
\(y_{\text {SDG }}=\left(\mathrm{n}_{2}+1\right)\left(2 \mathrm{y}_{\text {SDG }}-1\right)+\mathrm{ay}_{\text {SDG }}+\mathrm{by}_{\text {SDG }}\)
```



```
\(\mathrm{y}_{(\mathrm{CouAG}+\text { FeAG })}=2\left(\mathrm{n}_{2}+1\right) \mathrm{y}_{(\mathrm{CouAG}+\text { FeAG })}+\mathrm{ay}_{(\mathrm{CouAG}+\text { FeAG })}+\mathrm{b}_{\left(\mathrm{y}_{(\mathrm{CouAG}+\text { FeAG })}-1\right)}\)
```

For the average (or total) lignan macromolecule, the molar proportions of SDG, HMGA, and hydroxycinnamic acid glucosides were recalculated from Table 2: $y_{\text {SDG }}=0.433, \mathrm{y}_{\text {HMGA }}$ $=0.294$ and $y_{(\text {CouAG }+ \text { FeAG })}=0.273$. By trying to solve this set of equations, they appeared to be dependent, which resulted in a continuum of solutions only restricted by the constraints. In Fig. 7 the possible solutions are plotted.
According to Fig. 7, the average lignan macromolecule can be described as follows:
$1 \leq \mathrm{n}_{2}+1 \leq 3.1$
$0.6 \leq \mathrm{b} \leq 2.0$
$0 \leq \mathrm{a} \leq 0.7$
The average chain length was modeled as being maximally 3 SDG moieties $\left(\mathrm{n}_{2}=2.1\right)$ with a hydroxycinnamic acid glucoside moiety on most terminal positions. This average chain length is lower than the chain length of 5 SDG units determined by Kamal-Eldin et al. (2001). In that study, CouAG or FeAG were not considered as being terminal units.


Figure 7. Plot describing the average lignan macromolecule in terms of $n_{2}+1$ (amount of units SDG), b (amount of terminal CouAG+FeAG units), and a (amount of terminal HMGA units).

## Chain length distribution of the lignan macromolecule

Information about the chain length distribution can be obtained from the fractions obtained by GPC. In Table 4, the molar proportions (\%) of SDG and CouAG+FeAG per GPCfraction are given. Since no data about the content of HMGA in the fractions are available,

Chain length is determined by the incorporation of hydroxycinnamic acid glucosides
the lignan macromolecule should now be described by a combination of only SDG and CouAG + FeAG.
$\mathrm{LM}=\left(\mathrm{n}_{2}+1\right) \mathrm{SDG}+\mathrm{b}(\mathrm{CouAG}+\mathrm{Fe} A G)$
(equation 1b)
and
$\mathrm{Z}_{\mathrm{SDG}}+\mathrm{Z}_{(\text {(CouAG }+ \text { FeAG })}=1 \quad$ (equation 2 b )
in which $\left(n_{2}+1\right)=$ number of units SDG, $b=$ number of terminal hydroxycinnamic acid glucosides (CouAG + FeAG), and $z$ are the molar ratios of SDG and CouAG + FeAG. The value for $\mathrm{n}_{2}$ should be $\geq 0$, and $0 \leq \mathrm{b} \leq 2$. $\mathrm{z}_{\mathrm{SDG}}$ and $\mathrm{z}_{(\text {(CouAG }+ \text { FeAG) }}$ can be rewritten as follows by combining equations 1 b and 2 b :
$\mathrm{Z}_{\mathrm{SDG}}=\left(\mathrm{n}_{2}+1\right) /\left(\left(\mathrm{n}_{2}+1\right)+\mathrm{b}\right)$
$\mathrm{Z}_{\text {(CouAG }+ \text { FeAG) }}=\mathrm{b} /\left(\left(\mathrm{n}_{2}+1\right)+\mathrm{b}\right)$
or combined: $\left(\mathrm{n}_{2}+1\right)=\mathrm{Z}_{\mathrm{SDG}} / \mathrm{z}_{\text {(CouAG }+ \text { FeAG })} * \mathrm{~b} \quad$ (equation 4 b )
Also these equations were found to be dependent, giving a series of solutions limited by the constraints. In Table 4, the (dependent) ranges for $n_{2}$ (units of SDG) and b (amount of terminal hydroxycinnamic acid glucoside units) per GPC-fraction are given. Also the weighted average proportions of SDG and CouAG + FeAG (= (peak area * mol\% per fraction)/ (total area * mol\% of all fractions)) were calculated and used to model the weighted average values for $n_{2}$ and $b$. The weighted average composition of all GPCfractions resulted in a chain length of maximally 3 SDG units ( $\mathrm{n}_{2}=1.9$ ). This corresponds very well to the chain length found for the average lignan macromolecule.

Table 4. Characteristics of the GPC-fractions of the lignan macromolecule.

|  | GPC fraction |  |  |  |  |  |  |  | Weighted |
| :--- | :---: | :---: | :---: | :---: | :---: | :---: | :---: | ---: | ---: |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | average |
| Peak area (\%) | 2 | 5 | 16 | 27 | 26 | 15 | 7 | 2 | 100 |
| SDG (mol\%)* | 78 | 77 | 72 | 68 | 53 | 47 | 34 | 31 | 59 |
| HCAG (mol\%)* | 22 | 23 | 28 | 32 | 47 | 53 | 66 | 69 | 41 |
| $\mathrm{n}_{2}+1^{* *}$ | $1-7.1$ | $1-6.7$ | $1-5.1$ | $1-4.3$ | $1-2.3$ | $1-1.8$ | 1 | 1 | $1-2.9$ |
| $\mathrm{~b}^{\#}$ | $0.3-2$ | $0.3-2$ | $0.4-2$ | $0.5-2$ | $0.9-2$ | $1.1-2$ | 2 | 2 | $0.7-2$ |

* Molar fractions of SDG and CouAG+FeAG (=HCAG) are derived from Fig. 5 and are used to calculate $\mathrm{n}_{2}+1$ en b by solving equations 3 . SDG represents the molar fraction ( $\mathrm{mol} \%$ ) of SDG plus HDG.
** $n_{2}+1$ is the amount of SDG (plus HDG) units per macromolecule.
\# b is the amount hydroxycinnamic acid glucosides at the terminal positions.
For fractions 7 and 8 , the equations 3 b can only be solved when $\mathrm{b}=2$. For the other fractions, $b$ (and $n_{2}$ ) can vary. The maximal chain length of the lignan macromolecule from flaxseed hulls was found to be 7 SDG (or HDG; $n_{2}=6.1$ ) moieties with two hydroxycinnamic acid glucoside residues as terminal units $(b=2)$. These data fit well with the maximum found chain length of at least 5 SDG units as shown by MALDI-TOF MS.


## Chapter 4

## Conclusion

The average chain of the lignan macromolecule consists of three backbone units (SDG or HDG; $n_{2}=2$ ). The chain length varies between one and seven SDG (or HDG) moieties ( $0 \leq$ $n_{2} \leq 6$ ) per molecule. An example of an average lignan macromolecule is shown in Fig. 6B. The results obtained indicate that the hydroxycinnamic acid glucosides are most likely present at the terminal positions. These hydroxycinnamic acid glycosides seem to determine the chain length of the lignan macromolecule during biosynthesis.

## Experimental

## Extraction lignan macromolecule from flaxseed hulls

Lignan macromolecule was extracted from flaxseed hulls as described previously (Struijs et al., 2007). In short, 400 g of flaxseed hulls, kindly provided by Frutarom Netherlands B.V. (Veenendaal, The Netherlands), were extracted with 3 l of $n$-hexane. The lignan macromolecule was extracted from the defatted hulls by a three-step sequential extraction with $63 \%(\mathrm{v} / \mathrm{v})$ aq. EtOH at room temperature under continuous stirring. The extracts and the hulls were separated by filtration on a 595 round paper filter (Schleicher \& Schuell). From the combined extracts the EtOH was evaporated and the concentrated extract was lyophilized yielding the lignan macromolecule.

## Saponification of the lignan macromolecule

To release monomeric constituents, solutions of (fractions of) lignan macromolecule were fully saponified in 75 mM NaOH . The incubation volume was 1 ml , the concentration lignan macromolecule was $2 \mathrm{mg} / \mathrm{ml}$ or less. Saponification was performed at room temperature while stirring. After 24 h , the reaction was stopped by lowering the pH to 6.57.0 with HOAc.

## Sample clean up of (saponified) lignan macromolecule

Prior to analysis, low molecular weight polar material was removed from the lignan macromolecule extract or saponified samples by solid phase extraction (SPE; SepPak Vac, $20 \mathrm{cc} / 5 \mathrm{~g}, \mathrm{C} 18$ cartridge, Waters). Prior to loading samples onto the SPE cartridge, the EtOH concentration in the reaction mixture was reduced by diluting the samples with 25 ml water.
After activation of the SepPak cartridge first with MeOH and followed by water, the diluted saponified lignan macromolecule solution was loaded onto the cartridge. After a wash step with 1 column volume of water, lignans were eluted from the cartridge with 3 column volumes of MeOH of which the first column volume was discarded. The MeOH eluate was evaporated under a stream of air to a final volume of 1 ml .

## Reversed phase HPLC coupled on-line to mass spectrometry (RP-HPLC-MS)

Samples were analyzed on an X-Terra C18 MS column (Waters; $3.5 \mu \mathrm{~m}$ particle size, 4.6 x 150 mm ) with an X-Terra C18 MS guard column (Waters; $3.5 \mu \mathrm{~m}$ particle size, $4.6 \times 10$ mm ) run on a Thermo Separation Products HPLC system as previously described (Struijs et al., 2007). Samples were injected being dissolved in MeOH.
The molecular masses of the constituents of the macromolecule were determined on a Thermo Finnigan LCQ Classic coupled on-line to the analytical RP-HPLC. 10\% of the flow from the analytical RP-HPLC was directed to the MS. The MS was equipped with an ESI injector. Spectra were obtained in the negative ion mode over an $\mathrm{m} / \mathrm{z}$ range of 150-2000. The capillary temperature was $270{ }^{\circ} \mathrm{C}$, the ion spray voltage was set on 4.50 kV , the capillary voltage was -7.00 V , and helium was used as sheath gas. MS/MS analysis was performed with a normalized collision energy of $27 \%$.

## Gel permeation chromatography (GPC)

To obtain fractions of the lignan macromolecule with different molecular masses, aliquots of $100 \mu \mathrm{l}$ containing 2 mg lignan macromolecule (cleaned over SPE) /ml $63 \%$ (v/v) EtOH were injected on a similar HPLC system as described for RP-HPLC, equipped with a Tricorn Superdex Peptide 10/300 GL column (Amersham Bioscience; $10 \times 300-310 \mathrm{~mm}$, bed volume $=24 \mathrm{ml}$, optimum separation range $100-7000 \mathrm{Da}$ ). Fractions ( 0.8 ml ) were collected during three subsequent runs. The column was run isocratically at a flow rate of $0.8 \mathrm{ml} / \mathrm{min}$ with $40 \%(\mathrm{v} / \mathrm{v})$ aq. ACN $+0.1 \%(\mathrm{v} / \mathrm{v})$ TFA as eluent. The eluate was monitored at 280 nm . The included volume ( $\mathrm{V}_{\mathrm{inc}}=16.5 \mathrm{ml}$ ) was determined with water. No further calibration was performed since no suitable calibration compounds were available.
The same procedure was used to determine the molecular weight distribution of the GPCfractions isolated from the parental lignan macromolceule. The injection volume was $50 \mu$ l.

## Determination molar extinction coefficients of CouAG, FeAG, HDG and SDG

CouAG, FeAG, HDG and SDG were purified by preparative RP-HPLC as described previously (Struijs et al., 2007). Based on stock-solutions of about $10 \mathrm{mg} / \mathrm{ml}$, dilution series in MeOH were made. The absorbance at 280 nm of these dilutions was measured against MeOH in a 1 ml quartz cuvet. The molar extinction coefficients ( $\varepsilon$ ) were calculated using Abs $=\varepsilon * l *$ c in which Abs $=$ absorbance at $280 \mathrm{~nm}, \mathrm{l}=$ light path $=1 \mathrm{~cm}, \mathrm{c}=$ concentration (M).

## Determination HMGA by GC-MS

Lignan macromolecule ( $2 \mathrm{mg} / \mathrm{ml}$ in EtOH ) was fully saponified. Aliqouts ( 0.5 ml ) were spiked with $50 \mu$ g glutaric acid (GA) dissolved in water ( $50 \mu \mathrm{l}, 1 \mathrm{mg} / \mathrm{ml}$ ) as internal standard. A calibration curve was made with a concentration range of HMGA (Aldrich) starting from a $1 \mathrm{mg} / \mathrm{ml}$ stock-solution. The volume was increased to 5 ml with water and the pH was adjusted to $\mathrm{pH} 1.5-2.0$ with HCl . The samples were evaporated to dryness under a stream of air at $50^{\circ} \mathrm{C}$. To remove residual water, $100 \mu \mathrm{l}$ acetone was added and evaporated. Reaction vials were purged with $\mathrm{N}_{2}$.
To obtain TMS derivatives, to each dried sample 1 ml of HMDS:TMCS:pyridine (3:1:9; SIL-PREP Kit, Alltech) was added. After 2 h the derivatisation reaction was complete and the derivatized samples were analyzed on a GC 8000TOP system with an AS800 autosampler and FID-detection (Thermo Finnigan) equipped with a DB-1 column (J\&W; 30 $x 0.25 \times 1.0$ ). Injection volume was $1 \mu$. The temperature program was a follows: $t=0,80$ ${ }^{\circ} \mathrm{C} ; \mathrm{t}=0-12,80-325^{\circ} \mathrm{C}$ (linear increase); $\mathrm{t}=10-42,325^{\circ} \mathrm{C}$. Helium was used as a carrier gas at 100 kPa . The FID-detector was set on $350^{\circ} \mathrm{C}$.
GC-MS data were obtained by analyzing the derivatized samples on a Trace GC (Thermo Finnigan) coupled to a Polaris Q mass spectrophotometer. The settings of the GC were similar as describe above. MS data were collected in full scan mode between 5 and 25 min of the GC run, in the positive ion mode over a mass range of 50-400 Da. The temperature of the ion source was $250^{\circ} \mathrm{C}$. The auto-tune function was used to optimize the MS settings.

## Calculation of the molar proportions and the weight percentages

The molar proportions of the four phenolic compounds were calculated by dividing the molar equivalents of the individual compounds by the total molar equivalents. The molar

Chain length is determined by the incorporation of hydroxycinnamic acid glucosides
equivalents were determined by multiplying the molar extinction coefficients (Table 1) by the peak area under the RP-HPLC profiles of fully saponified lignan macromolecule (chromatograms not shown). The weight percentages were calculated based on the measured value for HMGA and the molar proportions of the phenolic constituents. HMGA was measured to contribute for $11.1 \%(\mathrm{w} / \mathrm{w})$ to the total lignan macromolecule. The four phenolic compounds accounted for the residual 88.9\% (w/w).

## MALDI-TOF MS of lignan macromolecule

For the determination of the molecular mass distribution, unsaponified lignan macromolecule was analyzed on a Ultraflex MALDI-TOF MS (Bruker Daltronics GmbH). As matrix 2,5-dihydroxybenzoic acid dissolved in $50 \%$ aq. ACN ( $15 \mathrm{mg} / \mathrm{ml}$ ) was used. Two $\mu \mathrm{l}$ of sample mixture consisting of $10 \mu \mathrm{l} 2 \mathrm{mg} / \mathrm{ml}$ unsaponified lignan macromolecule cleaned over SPE, $10 \mu \mathrm{l}$ matrix-solution, and $1 \mu \mathrm{l} 1 \mathrm{mM} \mathrm{NaOAc} \mathrm{pH} 5.0$ was spotted on a gold plate. The MALDI-TOF MS was calibrated with a mixture of maltodextrins (mass range 365-4092 Da). The system was used in the positive reflector mode.

## References

Boerjan, W., Ralph, J., Baucher, M., 2003. Lignin biosynthesis. Annu. Rev. Plant Biol. 54, 519-546.

Dabrowski, K.J., Sosulski, F.W., 1984. Composition of free and hydrolyzable phenolic acids in defatted flours of ten oilseeds. J. Agric. Food Chem. 32, 128-130.

Davin, L.B., Wang, H.B., Crowell, A.L., Bedgar, D.L., Martin, D.M., Sarkanen, S., Lewis, N.G., 1997. Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. Science 275, 362-366.

Eliasson, C., Kamal-Eldin, A., Andersson, R., Aman, P., 2003. High-performance liquid chromatographic analysis of secoisolariciresinol diglucoside and hydroxycinnamic acid glucosides in flaxseed by alkaline extraction. J. Chromatogr. A 1012, 151-159.

Ford, J.D., Huang, K.S., Wang, H.B., Davin, L.B., Lewis, N.G., 2001. Biosynthetic pathway to the cancer chemopreventive secoisolariciresinol diglucoside-hydroxymethyl glutaryl ester- linked lignan oligomers in flax (Linum usitatissimum) seed. J. Nat. Prod. 64, 1388-1397.

Hano, C., Martin, I., Fliniaux, O., Legrand, B., Gutierrez, L., Arroo, R.R.J., Mesnard, F., Lamblin, F., Laine, E., 2006. Pinoresinol-lariciresinol reductase gene expression and secoisolariciresinol diglucoside accumulation in developing flax (Linum usitatissimum) seeds. Planta 224, 1291-1301.

Johnsson, P., Kamal-Eldin, A., Lundgren, L.N., Aman, P., 2000. HPLC method for analysis of secoisolariciresinol diglucoside in flaxseeds. J. Agric. Food Chem. 48, 5216-5219.

Johnsson, P., Peerlkamp, N., Kamal-Eldin, A., Andersson, R.E., Andersson, R., Lundgren, L.N., Aman, P., 2002. Polymeric fractions containing phenol glucosides in flaxseed. Food Chem. 76, 207-212.

Kamal-Eldin, A., Peerlkamp, N., Johnsson, P., Andersson, R., Andersson, R.E., Lundgren, L.N., Aman, P., 2001. An oligomer from flaxseed composed of secoisolariciresinoldiglucoside and 3-hydroxy-3-methyl glutaric acid residues. Phytochemistry 58, 587-590.

Klosterman, H.J., Smith, F., 1954. The isolation of beta-hydroxy-beta-methylglutaric acid from the seed of flax (Linum-usitatissimum). J. Am. Chem. Soc. 76, 1229-1230.

Liggins, J., Grimwood, R., Bingham, S.A., 2000. Extraction and quantification of lignan phytoestrogens in food and human samples. Anal. Biochem. 287, 102-109.

Chain length is determined by the incorporation of hydroxycinnamic acid glucosides
Madhusudhan, B., Wiesenborn, D., Schwarz, J., Tostenson, K., Gillespie, J., 2000. A dry mechanical method for concentrating the lignan secoisolariciresinol diglucoside in flaxseed. Food. Sci. Technol. 33, 268-275.

Meagher, L.P., Beecher, G.R., Flanagan, V.P., Li, B.W., 1999. Isolation and characterization of the lignans, isolariciresinol and pinoresinol, in flaxseed meal. J. Agric. Food Chem. 47, 3173-3180.

Moss, G.P., 2000. Nomenclature of lignans and neolignans (IUPAC Recommendations 2000). Pure Appl. Chem. 72, 1493-1523.

Prasad, K., 2004. Lignan complex derived from flaxseed as hypercholesterolemic and antiatherosclerotic agent. International Patent US 6,673,773 B2.

Qiu, S.-X., Lu, Z.-Z., Luyengi, L., Lee, S.K., Pezzuto, J.M., Farnsworth, N.R., Thompson, L.U., Fong, H.H.S., 1999. Isolation and characterization of flaxseed (Linum usitatissimum) constituents. Pharm. Biol. 37, 1-7.

Sicilia, T., Niemeyer, H.B., Honig, D.M., Metzler, M., 2003. Identification and stereochemical characterization of lignans in flaxseed and pumpkin seeds. J. Agric. Food Chem. 51, 1181-1188.

Strandas, C., Kamal-Eldin, A., Andersson, R., Aman, P., 2008. Composition and properties of flaxseed phenolic oligomers. Food Chem. 110, 106-112.

Struijs, K., Vincken, J.-P., Verhoef, R., van Oostveen-van Casteren, W.H.M., Voragen, A.G.J., Gruppen, H., 2007. The flavonoid herbacetin diglucoside as a constituent of the lignan macromolecule from flaxseed hulls. Phytochemistry 68, 1227-1235.

Struijs, K., Vincken, J.-P., Verhoef, R., Voragen, A.G.J., Gruppen, H., 2008. Hydroxycinnamic acids are ester-linked directly to glucosyl moeities within the lignan macromolecule from flaxseed hulls. Phytochemistry 69, 1250-1260.
von Heimendahl, C.B.I., Schafer, K.M., Eklund, P., Sjoholm, R., Schmidt, T.J., Fuss, E., 2005. Pinoresinol-lariciresinol reductases with different stereospecificity from Linum album and Linum usitatissimum. Phytochemistry 66, 1254-1263.

Westcott, N.D., Muir, A.D., 1996. Process for extracting and purifying lignans and cinnamic acid derivatives from flaxseed. International Patent WO 9630468.

Youn, B.Y., Moinuddin, S.G.A., Davin, L.B., Lewis, N.G., Kang, C.H., 2005. Crystal structures of apo-form and binary/ternary complexes of Podophyllum secoisolariciresinol dehydrogenase, an enzyme involved in formation of health-protecting and plant defense lignans. J. Biol. Chem. 280, 12917-12926.

## Chapter 5 <br> Bacterial conversion of anhydrosecoisolariciresinol



Chapter 5


#### Abstract

Plant lignans are converted into bioactive mammalian lignans by the human intestinal flora. Anhydrosecoisolariciresinol (AHS) is formed during acid treatment of the plant lignan secoisolarisirecinol (SECO) or its diglucoside (SDG). During this reaction, the diol moiety of SECO is converted into a furan ring. In this study, it was investigated whether AHS could be fermented in a similar way and to a similar extent as SECO. AHS and SECO were demethylated in vitro by Peptostreptococcus productus, Eubacterium limosum and Clostridium methoxybenzovorans. These bacteria have been identified as members of the human intestinal flora or closely related species. The conversion of the diol structure of SECO into the furan ring in AHS did not influence the demethylation capability of the tested bacteria. Demethylated AHS and demethylated SECO were purified by preparative RP-HPLC, and subsequently subjected to fermentation with Eggerthella lenta, Clostridium scindens and Clostridium hiranonis. Eg. lenta efficiently dehydroxylated demethylated SECO to enterodiol, whereas the other bacteria showed no dehydroxylation activity. The results also clearly showed that the extent of dehydroxylation of demethylated AHS was much lower than that of demethylated SECO.


## Introduction

The main lignan from flaxseeds, secoisolariciresinol (SECO), is fermented into the mammalian lignans enterodiol (END) and enterolactone (ENL) by the human intestinal flora (Setchell et al., 1981; Wang et al., 2000). These mammalian lignans are thought to have estrogenic properties and are, therefore, suggested to contribute to the health effects of flaxseeds (Wang, 2002).
The lignans from flaxseeds are linked within an oligomeric structure called the lignan macromolecule (Kamal-Eldin et al., 2001; Struijs et al., 2007). Besides the diglucosidic form of SECO (secoisolariciresinol diglucoside; SDG), this lignan macromolecule is composed of the flavonoid herbacetin diglucoside (HDG), the hydroxycinnamic acid glucosides, coumaric acid glucoside (CouAG) and ferulic acid glucoside (FeAG), and the linker 3-hydroxy-3-methyl glutaric acid (HMGA) (Kamal-Eldin et al., 2001; Struijs et al., 2007).

During ingestion, lignans are released from the lignan macromolecule most likely by bacterial enzyme activity in the colon (Clavel et al., 2006a; Setchell et al., 2002). Although it is also possible that the brush border enzymes of the intestine play a role (Day et al., 1998; Nemeth et al., 2003). Bacteria belonging to the Bacteroides are good deglucosylating bacteria and also several Clostridia have been identified to release the glucosyl moieties from SDG to yield SECO (Clavel et al., 2006b). The fermentation of SECO in the colon can be divided into three steps: demethylation, dehydroxylation, and dehydrogenation (Fig. 1). Peptostreptococcus productus, Eubacterium callanderi, Eubacterium limosum and Bacteroides methylotrophicum have been identified as being involved in the demethylation reaction (Clavel et al., 2005; Clavel et al., 2006b; Wang et al., 2000). These bacteria all belong to the group of acetogenic bacteria (Clavel et al., 2006b; Drake et al., 2002). A strain isolated from feces, classified as Eggerthella lenta (previously called Eubacterium lentum (Kageyama et al., 1999)), was found to dehydroxylate previously demethylated compounds (Jin et al., 2007b; Wang et al., 2000). Clostridium scindens was also suggested to be able to dehydroxylate lignans, but only when co-incubated with P. productus (Clavel et al., 2006b). For the dehydrogenation of END into ENL, several Clostridia and Ruminococcus sp. have been described (Clavel et al., 2006b; Clavel et al., 2007; Jin et al., 2007a).

Acid treatment of the lignan macromolecule results in the hydrolysis of the glycosidic linkages and the release of SECO. Also anhydrosecoisolariciresinol (AHS) is formed under acidic conditions as a result of a dehydration reaction (Charlet et al., 2002; Mazur et al., 1996; Schöttner et al., 1997). The proportion of SECO converted into AHS is dependent on the acid concentration and the incubation time (Charlet et al., 2002). Since SECO and AHS show the same decoration of their phenolic rings, it is hypothesized that AHS can be fermented in the same way as SECO and that a rare type of mammalian lignan, enterofuran (ENF), can be formed. In Fig. 1 a tentative pathway for the fermentation of AHS is shown. So far, there are no reports describing the fermentation of AHS, and it is unknown whether the same bacteria as those involved in the fermentation of SECO can mediate its
conversion. As a first investigation towards the applicability of AHS as a potential bioactive component, the bacterial conversion of AHS into the enterolignan ENF was investigated.


Figure 1. Chemical structures and (putative) conversion pathway of SECO and AHS into the mammalian lignans END, ENL and ENF. a = bacterial demethylation, $\mathrm{b}=$ bacterial dehydroxylation, $\mathrm{c}=$ bacterial dehydrogenation, $\mathrm{d}=$ acid dehydration, ? = putative conversion.

## Results

## Preparation of AHS

AHS (2,3-bis(3-hydroxy-4-methoxybenzyl)-1,4-epoxybutane) was synthesized from fully saponified lignan macromolecule (Fig. 2A). RP-HPLC analysis of the untreated saponified lignan macromolecule showed the presence of CouAG, FeAG, HDG and SDG, besides some unidentified peaks (Fig. 2A). SDG was hydrolyzed completely in 2 h under acidic conditions, as the peak of SDG was absent in the RP-HPLC profile of the incubated samples. A peak assigned as AHS (Table 1) was formed during acid treatment. During the reaction, AHS became partially insoluble in water and the pellet was resolubilized in MeOH to recover all AHS. After 2 h of incubation, a small amount of SECO (assigned based on MS data as shown in Table 1) was still present, which means that the reaction did not reach an endpoint. The reaction was stopped after 2 h because it is known that for longer incubation times the amount of AHS decreases, resulting in a lower yield (Charlet et al., 2002).


Figure 2. (A) RP-HPLC profiles of AHS formation by acid treatment of saponified lignan macromolecule before and after treatment. (B) Time curves of the conversion of SDG via SECO into AHS under acidic conditions. AHS represents both the AHS in the pellet as the AHS in the supernatant.

The formation of AHS was followed in time as shown in Fig. 2B. The amounts of AHS present in the pellet and in the supernatant were summed. After 2 h of incubation, the amount of AHS was still increasing showing that its formation was still predominant over its degradation.
Also CouAG, FeAG, and HDG reacted under influence of the acidic conditions as observed by the decline in peak areas (Fig. 2A). However, a distinct product peak could not be identified. To be able to test the microbial conversion of AHS, it was purified by preparative RP-HPLC (data not shown). The purity of the AHS obtained was $95 \%$ based on peak area on analytical RP-HPLC.

## Preparation of SECO

To be able to compare the bacterial conversion of AHS with that of SECO, also SECO was prepared. SECO (2,3-bis(3-hydroxy-4-methoxybenzyl)butane-1,4-diol) was obtained by deglucosylation of fully saponified lignan macromolecule as shown in Fig. 3. RP-HPLCMS analysis (Fig. 3A) revealed that SDG and the hydroxycinnamic acid glucosides were fully deglucosylated within 24 h and that SECO ( $\mathrm{m} / \mathrm{z}=361.3[\mathrm{M}-\mathrm{H}]^{-}$), $p$-coumaric acid (CouA; $m / z=163.1[\mathrm{M}-\mathrm{H}]^{-}$) and ferulic acid ( $\mathrm{FeA} ; m / z=192.9[\mathrm{M}-\mathrm{H}]^{-}$) were formed. As can be seen in Fig. 3B, the deglucosylation of SDG was complete after 20 h of incubation. SECO was purified from the deglucosylated saponified lignan macromolecule by preparative RP-HPLC (data not shown). The SECO obtained was $96 \%$ pure, based on peak area on analytical RP-HPLC.


Figure 3. (A) RP-HPLC profile of fully saponified lignan macromolecule before ( $\mathrm{t}=0 \mathrm{~h}$ ) and after deglucosylation ( $\mathrm{t}=24 \mathrm{~h}$ ) by Rapidase $\mathrm{Liq}^{+}$. (B) Time curves of the deglucosylation of SDG into SECO.

## Demethylation of AHS and SECO

AHS and SECO were incubated with P. productus, E. limosum and Clostridium methoxybenzovorans. P. productus and E. limosum were chosen since they are known to demethylate lignans (Clavel et al., 2006b; Wang et al., 2000). Cl. methoxybenzovorans was chosen as a representative of the Clostridia sp. (Clavel et al., 2006b) because it produces an $O$-demethylase, which is active against a broad range of methoxylated aromatic compounds (Mechichi et al., 1999).
In Fig. 4A, the RP-HPLC profiles of the fermentation of AHS by P. productus after 0, 8 and 24 h of incubation are shown. At the beginning of the incubation, only AHS was present eluting at a RT of 33.1 min . After 8 hours of incubation, AHS, mono-demethylated AHS (RT $30.4 \mathrm{~min} ; 2$-(3,4-dihydroxybenzyl)-3-(3-hydroxy-4-methoxybenzyl)-1,4-epoxybutane (= methoxy-hydroxyENF = MHENF)) and di-demethylated AHS (RT 25.1 min ; 2,3-bis(3,4-dihydroxybenzyl)-1,4-epoxybutane (= dihydroxyENF = DHENF)) were detected. After 24 h of incubation (Fig. 4A), only a single peak with RT 25.1 min, assigned as DHENF, was observed. Assignment was based on MS and MS ${ }^{2}$ data as shown in Table 1. The fermentation of AHS by P. productus was followed in time (Fig. 4B). Also the growth, indicated by the $\mathrm{OD}_{600 \mathrm{~nm}}$ values, was measured (Fig. 4B). After an initial adjustment period
of some hours, the fermentation proceeded quickly. Already before growth was completed, more than $50 \%$ of the AHS was converted into consecutively MHENF and DHENF. A few hours after maximal $\mathrm{OD}_{600 \mathrm{~nm}}$ was reached ( $\sim 12 \mathrm{~h}$ of incubation) demethylation of AHS was complete.


Figure 4. (A) RP-HPLC profiles of the fermentation of AHS by P. productus after 0,8 and 24 h of incubation (For assignments, see Table 1). (B) Demethylation of AHS by P. productus in time. The growth was monitored by measuring the $\mathrm{OD}_{600 \mathrm{~nm}}$.


Figure 5. (A) RP-HPLC profiles of the fermentation of SECO by P. productus after 0,8 and 24 h of incubation (For assignments, see Table 1). (B) Demethylation of SECO by $P$. productus in time. The growth was monitored by measuring the $\mathrm{OD}_{600 \mathrm{~nm}}$.

The demethylation of AHS by P. productus was compared with that of SECO by this same organism. The fermentation products were analyzed by RP-HPLC-MS (Fig. 5A and Table 1). The fermentation of SECO by $P$. productus in time is shown in Fig. 5B. Monodemethylated SECO (RT 20.0 min; 2-(3,4-dihydroxybenzyl)-3-(3-hydroxy-4-methoxybenzyl)butane-1,4-diol (= methoxy-hydroxyEND = MHEND)) was formed first, followed by the second demethylation step, which yielded di-demethylated SECO (RT 16.8 min; 2,3-bis(3,4-dihydroxybenzyl)butane-1,4-diol (= dihydroxyEND = DHEND)). After 10

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$h$ of incubation the conversion was complete (Fig. 5B). These results showed that the demethylation of AHS proceeded similarly as the demethylation of SECO (Fig. 4 and 5).

Tabel 1. MS data of fermentation products of AHS and SECO.

| RT HPLC | $m / \mathrm{z}$ - ratio <br> $[\mathrm{M}-\mathrm{H}]$ | MS $^{2}$ fragments | Mw | Assignment |
| :---: | :---: | :---: | :---: | :---: |
| 23.2 | $361.2 ; 721.4$ | $313.3 ; 269.2 ; 164.7$ | 362.2 | SECO |
| 20.0 | 347.3 | $332.1 ; 317.2 ; 287.1 ; 164.9$ | 348.2 | MHEND |
| 16.8 | 333.3 | $315.0 ; 285.1 ; 259.4 ; 181.0$ | 334.1 | DHEND |
| 22.4 | 317.3 | $286.7 ; 268.9 ; 258.0 ; 164.3$ | 318.1 | HEND |
| 28.2 | 301.2 | $283.1 ; 253.3 ; 241.1 ; 174.8$ | 302.1 | END |
| 33.3 | 343.2 | $328.1 ; 301.1 ; 239.8 ; 148.9$ | 344.2 | AHS |
| 30.8 | 329.2 | $314.1 ; 287.3 ; 247.0 ; 164.1$ | 330.1 | MHENF |
| 25.9 | 315.3 | $298.4 ; 287.2 ; 269.6 ; 163.1$ | 316.1 | DHENF |
| 31.3 | 299.3 | $271.4 ; 218.2 ; 174.9$ | 300.1 | HENF |
| 33.9 | 283.3 | No MS $^{2}$ data collected | 284.1 | ENF |

Table 2. Demethylation of AHS and dehydroxylation of DHENF.

| Strain | demethylation AHS |  |  | dehydroxylation DHENF |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | $\begin{aligned} & \mathrm{t}_{1 / 2}^{1} \\ & \text { (h) } \\ & \hline \end{aligned}$ | $\begin{aligned} & \mathrm{t}_{\text {full }}{ }^{2} \\ & \text { (h) } \\ & \hline \end{aligned}$ | $\begin{gathered} \mathrm{t}_{\text {max }}^{3} \\ \mathrm{OD}_{600 \mathrm{~mm}}(\mathrm{~h}) \\ \hline \end{gathered}$ | $\mathrm{t}_{1 / 2}^{1}$ <br> (h) | $\begin{aligned} & \mathrm{t}_{\text {full }}{ }^{2} \\ & \text { (h) } \\ & \hline \end{aligned}$ | $\begin{gathered} \mathrm{t}_{\text {max }}^{3} \\ \mathrm{OD}_{600 \mathrm{~mm}}(\mathrm{~h}) \\ \hline \end{gathered}$ |
| P. productus | 10 | 12 | 10 | - ${ }^{4}$ | - | ND ${ }^{5}$ |
| E. limosum | 30 | 38 | 30 | - | - | ND |
| Cl. methoxybenzovorans | 12 | 16 | 18 | - | - | ND |
| Eg. lenta | $\mathrm{ND}^{5}$ | ND | ND | > 72 | > 72 | 7 |

${ }^{1}$ incubation time at which half of AHS or DHENF was converted.
${ }^{2}$ incubation time at which conversion was complete.
${ }^{3}$ incubation time at which maximum $\mathrm{OD}_{600 \mathrm{~nm}}$ was reached.
${ }^{4}$ no conversion detected; $\mathrm{t}_{1 / 2}$ and $\mathrm{t}_{\text {full }}$ could not be determined.
${ }^{5} \mathrm{ND}=$ not determined.
Table 3. Demethylation of SECO and dehydroxylation of DHEND.

| Strain | demethylation SECO |  |  | dehydroxylation DHEND |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | $\mathrm{t}_{1 / 2}^{1}$ <br> (h) | $\mathrm{t}_{\text {full }}{ }^{2}$ (h) | $\begin{gathered} \mathrm{t}_{\text {max }}^{3} \\ \mathrm{OD}_{600 \mathrm{om}}(\mathrm{~h}) \end{gathered}$ | $\mathrm{t}_{1 / 2}^{1}$ <br> (h) | $\mathrm{t}_{\text {full }}^{2}$ <br> (h) | $\begin{gathered} \mathrm{t}_{\text {max }}^{3} \\ \mathrm{OD}_{600 \mathrm{~nm}}(\mathrm{~h}) \end{gathered}$ |
| P. productus | 8 | 10 | 14 | - | - | ND ${ }^{5}$ |
| E. limosum | 16 | 20 | 24 | - | - | ND |
| Cl. methoxybenzovorans | 22 | 34 | 26 | - | - | ND |
| Cl. scindens | - ${ }^{4}$ | - | ND ${ }^{5}$ | - | - | ND |
| Cl. hiranonis | - | - | ND | - | - | ND |
| Eg. lenta | - | - | ND | 39 | 54 | 8 |

${ }^{1}$ incubation time at which half of SECO or DHEND was converted.
${ }^{2}$ incubation time at which conversion was complete.
${ }^{3}$ incubation time at which maximum $\mathrm{OD}_{600 \mathrm{~nm}}$ was reached.
${ }^{4}$ no conversion detected; $t_{1 / 2}$ and $t_{\text {full }}$ could not be determined.
${ }^{5} \mathrm{ND}=$ not determined.

For the other bacterial strains, similar results were obtained as for $P$. productus (Table 2 and 3). Although the absolute rates differed per strain, for all strains the time required for demethylation of AHS or SECO coincided more or less with the time required to reach the maximum $\mathrm{OD}_{600 \mathrm{~nm}}$. For E. limosum and Cl . methoxybenzovorans some differences in conversion time between demethylation of AHS and SECO were observed. These differences are most likely caused by batch differences.
Also the bacteria selected for their dehydroxylating capability (Eg. lenta, Cl. scindens and Cl. hiranonis) were tested for their demethylating capability, but they did not show any conversion (Table 3). The growth of the bacteria was not affected by the MeOH added, because similar growth curves were obtained for bacteria grown with or without MeOH in a control experiment (data not shown).
To obtain DHENF and DHEND for dehydroxylation experiments, AHS and SECO were demethylated, extracted and purified by preparative RP-HPLC (data not shown). As determined based on peak area of analytical RP-HPLC, the DHENF and DHEND obtained were more than $95 \%$ pure.

## Dehydroxylation of demethylated AHS and SECO

The second step in the fermentation of lignans is a dehydroxylation step. Eg. lenta was used since it was previously shown to dehydroxylate DHEND (Wang et al., 2000). In Fig. 6, the RP-HPLC profile of DHENF incubated for 72 h by Eg. lenta is shown. Peaks were assigned based on MS and MS ${ }^{2}$ data (Table 1). Only a small proportion of the DHENF (RT 25.9 min ) was dehydroxylated; two minor peaks corresponding to hydroxyENF (RT 31.3 min ; 2-(3-hydroxybenzyl)-3-(3,4-dihydroxybenzyl)-1,4-epoxybutane (= HENF)) and ENF (RT 33.8; 3,4-bis(3-hydroxybenzyl)-1,4-epoxybutane) were found.


Figure 6. RP-HPLC profile of demethylated AHS (DHENF) incubated for 72 h with Eg. lenta (For assignments, see Table 1).

To verify whether the bacterial strain was active, also DHEND was incubated with Eg. lenta. The RP-HPLC profiles are shown in Fig. 7A and the development of the intermediates in time is shown in Fig. 7B. After 50 h of incubation, DHEND was fully
converted into END (RT 28.2 min ; 3,4-bis(3-hydroxybenzyl)butane-1,4-diol), showing that the Eg. lenta DSM2243 was capable of dehydroxylating lignans.
Cl. scindens and Cl. hiranonis were found to be unable to dehydroxylate DHEND. These bacteria were selected because previous research showed that Cl. scindens could dehydroxylate SECO in co-incubation with $P$. productus and because of the capacity of these bacteria to dehydroxylate several bile acids (Clavel et al., 2006b; Kitahara et al., 2001a; Kitahara et al., 2001b).


Figure 7. (A) RP-HPLC profiles of the dehydroxylation of demethylated SECO (DHEND) by Eg. lenta after 0,30 and 60 h of incubation (For assignments, see Table 1). (B) Development of the dehydroxylation of DHEND in time. Growth was monitored by measuring the $\mathrm{OD}_{600 \mathrm{~nm}}$.

## Discussion

AHS is present in only a few natural sources (Fang et al., 1989; Yamamoto et al., 2004), but it can be formed during acid treatment of SECO or SDG (Charlet et al., 2002; Schöttner et al., 1997). While END and ENL are reported in human plasma or urine regularly, there is only a single study, which suggests that ENF might be present in human urine (Liggins et al., 2000). Therefore, the effects of AHS and its suggested mammalian form, ENF, are still unclear. As a first investigation towards the applicability of AHS as a bioactive component, this study investigated the fermentability of AHS.

## Demethylation of AHS

This study showed that AHS was demethylated in a similar way as SECO. This indirectly showed that the presence of a diol or furan structural element does not change the demethylating efficiency of the bacterial strains tested.
Cl. methoxybenzovorans was shown to demethylate both AHS and SECO. Although it has been reported that the $O$-demethylase of $C l$. methoxybenzovorans was active against a broad range of differently substituted aromatic compounds (Mechichi et al., 1999), this is the first time Cl. methoxybenzovorans was shown to demethylate lignans. The $O$-demethylase of $E$. limosum has a more narrow substrate specificity, since it was only active against methoxyl groups at the meta-postion (DeWeerd et al., 1988). The result that E. limosum can demethylate both AHS and SECO suggests that the structure of the aliphatic part of the molecule does not influence the capability of $E$. limosum to demethylate.
The demethylating bacteria $P$. productus, E. limosum and Cl. methoxybenzovorans all belong to the acetogenic bacteria (Drake et al., 2002). Acetogenic bacteria are anaerobic bacteria capable of using $\mathrm{CO}_{2}$ as their sole carbon source (Diekert, 1992). Also other onecarbon sources, as aromatic methoxy groups, can be used for this purpose (Muller et al., 2004). Therefore, it is expected that more members of the acetogenic baceria are capable of demethylating lignans. Cl. scindens and Cl. hiranonis do not have the capability do demethylate. This was also not expected, since they do not belong to the acetogenic bacteria.

## Dehydroxylation of AHS

The capability of bacteria to demethylate is widespread among different genera. The capability to dehydroxylate seems to be less common (or less studied). In contrast to demethylation, the advantages for the bacteria to dehydroxylate are less clear. It has been suggested that the reaction can be used for energy conservation (Brauman et al., 1998). Besides, it has been hypothesized that dehydroxylation is the first step in the degradation of phenolic compounds into organic acids (Brauman et al., 1998; Muller and Schink, 2000).

This study showed for the first time that DHENF is dehydroxylated by Eg. lenta, albeit to a certain extent, and that HENF and ENF can be formed. END was formed by Eg. lenta to a much higher extent, as shown in this and previous studies (Clavel et al., 2006b; Jin et al., 2007b; Wang et al., 2000). The growth and conversion rates found for the dehydroxylation

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reaction of DHEND in this study corresponded to the conversion rates found previously (Jin et al., 2007b; Wang et al., 2000).
These differences in extent of dehydroxylation between DHENF and DHEND suggest that the dehydroxylase has a higher substrate specificity than the $O$-demethylases, although differences in the amounts of enzyme produced, or substrate uptake can not be excluded. The structure of the aliphatic part of the molecule (diol in case of DHEND and furan ring in case of DHENF) is hypothesized to (partially) determine the substrate specificity.

In conclusion, this study showed that AHS can be fermented into ENF but at a much lower rate than SECO into END. Especially the dehydroxylation reaction was found to be much slower for the conversion of demethylated AHS into ENF than of demethylated SECO into END. The demethylation reaction was not influenced by the presence of a furan ring instead of a diol structure.

## Experimental

## Bacterial strains

Peptostreptococcus productus (DSMZ 2950), Clostridium methoxybenzovorans (DSMZ 12182), Clostridium scindens (DSMZ 5676), Clostridium hiranonis (DSZM 13275) and Eggerthella lenta (DSMZ 2243) were purchased at the Deutsche Sammlung von Mikroorganismun und Zellkulturen GmbH (Braunschwig, Germany).
Eubacterium lentum (LMG P-23546), selected for growth on 8-prenylnaringenin (Possemiers et al., 2005), was obtained from the Laboratory of Microbial Ecology and Technology of Ghent University (Ghent, Belgium).
The flaxseed hulls, which were used as a source of lignans, were kindly provided by Frutarom Netherlands B.V. (Veenendaal, The Netherlands).

## Fermentation of lignans

Brain heart infusion broth (BHI; Becton, Dickinson and Company) was prepared as indicated by the manufacturer. L-cysteinhydrochloride-monohydrate (Merck) was added to a concentration of 0.5 \% (w/v). Resazurine (Riedel-de Haen) was added as redox-indicator ( ~ 4 ppm).
Flasks of 100 ml were filled with 50 ml BHI medium. Prior to closure of the bottles, purified lignan, dissolved in 0.5 ml MeOH , was added. In the incubations, which were used to follow the conversion in time, concentrations of 0.2 mg lignan $/ \mathrm{ml}$ medium were used. To obtain material for further experiments, maximally 1 mg lignan $/ \mathrm{ml}$ medium was used. The medium was made anaerobic by flushing with nitrogen ( 8 cycles between -0.7 bar and 0.6 bar), and sterilized ( $20 \mathrm{~min}, 144{ }^{\circ} \mathrm{C}$ ). Media were inoculated with 5 ml of a full-grown, 2 day old, bacterial culture in BHI-medium (without lignans). Two batches, which were inoculated 10 h after each other, were used to be able to follow the fermentation during 24 h. Samples were incubated at $37^{\circ} \mathrm{C}$ in the dark without shaking. Samples of 1 ml were taken regularly. $\mathrm{OD}_{600 \mathrm{~nm}}$ was measured to monitor bacterial growth. The reaction was stopped by boiling the samples for 10 min .
Lignans were extracted from the fermentation samples (including the cells) by a three step liquid-liquid partitioning extraction with ethylacetate (1:3 (v/v)). The ethylacetate fractions were pooled, the ethylacetate evaporated and the extracted lignans were redissolved in MeOH prior to analysis on RP-HPLC-MS.

## Formation of AHS

AHS was prepared by acid treatment of fully saponified lignan macromolecule. Fully saponified lignan macromolecule has been shown previously to consist of 4 major phenolic constituents identified based on RP-HPLC-MS as SDG, CouAG, FeAG, and HDG (Struijs et al., 2007). Fully saponified lignan macromolecule was obtained as described previously (Struijs et al., 2007). To a 2 mg saponified lignan macromolecule $/ \mathrm{ml}$ water solution, HCl was added to a final concentration of 2 M . The solution was incubated at $100^{\circ} \mathrm{C}$ for 2 h . Every 15 min a sample ( 1 ml ) was taken to be able to follow the reaction in time, the rest was used to obtain AHS for fermentation studies. The reaction was stopped by adding NaOH to pH 6.5 while cooling on ice. During the reaction a pellet was formed. The pellet

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and supernatant were separated by centrifugation (for 1 ml samples: $15,700 \mathrm{~g}, 4^{\circ} \mathrm{C}, 10$ min ; for larger volumes: $28,100 \mathrm{~g}, 4^{\circ} \mathrm{C}, 30 \mathrm{~min}$ ).
For the samples of the time course, the pellet was resuspended in 1 ml MeOH and analyzed by analytical RP-HPLC. The aqueous supernatant was cleaned by SPE as described previously (Struijs et al., 2007). The MeOH-eluate of SPE was concentrated to 1 ml and analyzed by analytical RP-HPLC.
The pellet, which was obtained after centrifugation of the aqueous reaction mixture used to obtain higher amounts of AHS, was resuspended in an equal volume MeOH as the original aqueous volume. This resolubilized pellet was centrifuged ( $28,100 \mathrm{~g}, 4^{\circ} \mathrm{C}, 30 \mathrm{~min}$ ). The aqueous supernatant (obtained after centrifugation of the aqueous reaction mixture) was cleaned using SPE as described previously (Struijs et al., 2007). Prior to lyophilization, the MeOH was evaporated from the resolubilized pellet and from the MeOH-eluate from SPE. For fermentation purposes, the AHS was purified by preparative RP-HPLC (see below).

## Formation of SECO by enzymatic deglucosylation

Saponified lignan macromolecule was obtained as described previously (Struijs et al., 2007). To 2 mg saponified lignan macromolecule $/ \mathrm{ml} 50 \mathrm{mM} \mathrm{NaOAc}$ buffer $\mathrm{pH} 5.0,5 \mu \mathrm{l}$ Rapidase $\mathrm{Liq}^{+}$(DSM Food Specialties, Delft, The Netherlands) was added and the reaction mixture was incubated for 24 h at $35^{\circ} \mathrm{C}$. The enzymes were inactivated by boiling. SECO was extracted from the reaction mixture by SPE as described previously (Struijs et al., 2007), and was purified by preparative RP-HPLC.

## Analytical RP-HPLC-MS

Samples ( $20 \mu \mathrm{l}$ ) were analyzed on an X-Terra C18 MS column (Waters; $3.5 \mu \mathrm{~m}$ particle size, $4.6 \times 150 \mathrm{~mm}$ ) with an X-Terra C18 MS guard column (Waters; $3.5 \mu \mathrm{~m}$ particle size, $4.6 \times 10 \mathrm{~mm}$ ) run on a Thermo Separation Products HPLC system equipped with a membrane degasser, P4000 pump, AS3000 autosampler, and UV3000 detector. Water and acetonitrile (ACN), both acidified with $0.1 \%(\mathrm{v} / \mathrm{v}) \mathrm{HOAc}$, were used as eluents. The flow rate was $0.7 \mathrm{ml} / \mathrm{min}$. The following gradient was applied: 0-25 min, linear from $10 \%$ to $30 \%$ ACN; 25-30 min, linear to $50 \%$ ACN; 30-40 min, isocratic on 50\% ACN; 40-42 min, linear to $100 \%$ ACN; 42-47 min, isocratic on $100 \%$ ACN; 47-50 min, linear to $10 \%$ ACN; $50-60 \mathrm{~min}$, isocratic on $10 \%$ ACN. The eluate was monitored at 280 nm , and by mass spectroscopy on a Thermo Finnigan LCQ Classic equipped with an ESI-MS injector. One tenth of the flow from the analytical RP-HPLC was directed to the MS. Spectra were obtained in the negative ionization mode over an $m / z$-range of 150-2000 Da. The capillary temperature was $270{ }^{\circ} \mathrm{C}$, the capillary voltage was -7.00 V , the ion spray voltage was set on 4.50 kV and helium was used as sheath gas. $\mathrm{MS}^{2}$ analysis was performed with a normalized collision energy of $27 \%$.

## Purification of lignans by (semi)preparative RP-HPLC

In order to obtain purified AHS and SECO for fermentation studies, they were purified by preparative RP-HPLC on an X-Terra C18 MS column (Waters; $5 \mu \mathrm{~m}$ particle size, $50 \times 100$ mm , OBD) with an X-Terra C18 MS guard column (Waters; $5 \mu \mathrm{~m}$ particle size, 19 x 10 mm ) ran on a Waters preparative HPLC system equipped with a 2525 pump, 2767 sample manager, Fluid Organizer and 2996 photodiode array detector. For purification of
fermentation products, the samples were separated on a semi-preparative X-Terra C18 MS column (Waters; $5 \mu \mathrm{~m}$ particle size, $29 \times 150 \mathrm{~mm}$, OBD) with an X-Terra C18 MS guard column (Waters; $5 \mu \mathrm{~m}$ particle size, $19 \times 10 \mathrm{~mm}$ ). Water and acetonitrile (ACN), both acidified with $0.01 \% ~(\mathrm{v} / \mathrm{v}$ ) TFA, were used as eluents. Since the solubility of lignans in water is low and to avoid long loading times, a protocol called "at column dilution" was used to be able to load the samples dissolved in $100 \% \mathrm{MeOH}$. Sample was injected in a flow of $100 \%$ ACN delivered by a loading pump (P4000 Thermo Separation Products).
In case of preparative RP-HPLC, during the first 4.5 min the sample was loaded under a flow rate of $4 \mathrm{ml} / \mathrm{min}$ provided by the loading pump, while the 2525 pump was running at $36 \mathrm{ml} / \mathrm{min}$ at $100 \%$ water. The flows of the 2525 pump and the loading pump were mixed via a T-piece just before the column. Between 4.5 and 5 min , the flow rate of the 2525 pump increased to $80 \mathrm{ml} / \mathrm{min}$, the proportion ACN in the eluent increased to $10 \%(\mathrm{v} / \mathrm{v})$, and the loading pump stopped. Between 5 and 42 min , the following gradient was run at a flow rate of $80 \mathrm{ml} / \mathrm{min}$ : 5-21.6 min, linear to $30 \% ~(\mathrm{v} / \mathrm{v}$ ) ACN; 21.6-25 min, linear to $50 \% ~(\mathrm{v} / \mathrm{v}$ ) ACN; 25-31.6 min, isocratic on $50 \%(\mathrm{v} / \mathrm{v})$ ACN; 31.6-32.3 min, linear to $100 \%$ ACN; 32.335 min , isocratic on $100 \% \mathrm{ACN}$; 35-38.5 min, linear to $10 \%(\mathrm{v} / \mathrm{v}) \mathrm{ACN}$; 38.5-41.6 min, isocratic on $10 \%(\mathrm{v} / \mathrm{v}) \mathrm{ACN}$.
In case of semi-preparative RP-HPLC, the sample loading pump applied a flow rate of 1.2 ml ACN/min during the complete run ( 65 min ). The starting condition of the 2525 pump was $100 \%$ water. The flows of the 2525 pump and the loading pump were mixed via a Tpiece just before the column so that at the start of the run, $10 \%$ ACN was applied on the column. The flow rate of the 2525 pump was $10.8 \mathrm{ml} / \mathrm{min}$ during the complete run. The following elution profile was applied by the 2525: 0-7 min, isocratic on $100 \%$ water, 7-32 min , linear to $22 \%(\mathrm{v} / \mathrm{v}) \mathrm{ACN}$; 32-37 min, linear to $44 \%(\mathrm{v} / \mathrm{v}) \mathrm{ACN}$; 37-47 min, isocratic on $44 \% \mathrm{ACN}$; 47-50 min, linear to $100 \%$ ACN; 50-54 min, isocratic on $100 \%$ ACN; 54-57 min , linear to $100 \%$ water, $57-65 \mathrm{~min}$, isocratic on $100 \%$ water.
Fractions were collected based on the UV-response at 280 nm (leading edge gradient $=5 \%$, peak terminates when valley $=0$ ). Appropriate fractions were pooled, as were the corresponding pools from subsequent runs. ACN was evaporated and the fractions were lyophilized.

## References

Brauman, A., Muller, J.A., Garcia, J.L., Brune, A., Schink, B., 1998. Fermentative degradation of 3-hydroxybenzoate in pure culture by a novel strictly anaerobic bacterium, Sporotomaculum hydroxybenoicum gen. nov., sp. nov. Int. J. Syst. Bacteriol. 48, 215-221.

Charlet, S., Bensaddek, L., Raynaud, S., Gillet, F., Mesnard, F., Fliniaux, M.A., 2002. An HPLC procedure for the quantification of anhydrosecoisolariciresinol. Application to the evaluation of flax lignan content. Plant Physiol. Biochem. 40, 225-229.

Clavel, T., Henderson, G., Alpert, C.A., Philippe, C., Rigottier-Gois, L., Dore, J., Blaut, M., 2005. Intestinal bacterial communities that produce active estrogen-like compounds enterodiol and enterolactone in humans. Appl. Environ. Microbiol. 71, 6077-6085.

Clavel, T., Borrmann, D., Braune, A., Dore, J., Blaut, M., 2006a. Occurrence and activity of human intestinal bacteria involved in the conversion of dietary lignans. Anaerobe 12, 140147.

Clavel, T., Henderson, G., Engst, W., Dore, J., Blaut, M., 2006b. Phylogeny of human intestinal bacteria that activate the dietary lignan secoisolariciresinol diglucoside. FEMS Microbiol. Ecol. 55, 471-478.

Clavel, T., Lippman, R., Gavini, F., Dore, J., Blaut, M., 2007. Clostridium saccharogumia sp nov and Lactonifactor longoviformis gen. nov., sp nov., two novel human faecal bacteria involved in the conversion of the dietary phytoestrogen secoisolariciresinol diglucoside. Syst. Appl. Microbiol. 30, 16-26.

Day, A.J., DuPont, M.S., Ridley, S., Rhodes, M., Rhodes, M.J.C., Morgan, M.R.A., Williamson, G., 1998. Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver beta-glucosidase activity. FEBS Lett. 436, 71-75.

DeWeerd, K.A., Saxena, A., Nagle, D.P., Jr., Suflita, J.M., 1988. Metabolism of the 18Omethoxy substituent of 3-methoxybenzoic acid and other unlabeled methoxybenzoic acids by anaerobic bacteria. Appl. Environ. Microbiol. 54, 1237-1242.

Diekert, G., 1992. The Acetogenic Bacteria. In: Balows, A., Truper, H.G., Dworkin, M., Harder, W., Schleifer, K.H. (Eds.), The Prokaryotes. Spinger-Verlag, New York, pp. 517533.

Drake, H.L., Kusel, K., Matthies, C., 2002. Ecological consequences of the phylogenetic and physiological diversities of acetogens. Antonie Van Leeuwenhoek 81, 203-213.

Fang, J.M., Hsu, K.C., Cheng, Y.S., 1989. Lignans from leaves of Calocedrus-Formosana. Phytochemistry 28, 3553-3555.

Jin, J.S., Kakiuchi, J., Hattori, M., 2007a. Enantioselective oxidation of enterodiol to enterolactone by human intestinal bacteria. Biol. Pharm. Bull. 30, 2204-2206.

Jin, J.S., Zhao, Y.F., Nakamura, N., Akao, T., Kakiuchi, N., Min, B.S., Hattori, M., 2007b. Enantioselective dehydroxylation of enterodiol and enterolactone precursors by human intestinal bacteria. Biol. Pharm. Bull. 30, 2113-2119.

Kageyama, A., Benno, Y., Nakase, T., 1999. Phylogenetic evidence for the transfer of Eubacterium lentum to the genus Eggerthella as Eggerthella lenta gen. nov., comb. nov. Int. J. Syst. Bacteriol. 49, 1725-1732.

Kamal-Eldin, A., Peerlkamp, N., Johnsson, P., Andersson, R., Andersson, R.E., Lundgren, L.N., Aman, P., 2001. An oligomer from flaxseed composed of secoisolariciresinoldiglucoside and 3-hydroxy-3-methyl glutaric acid residues. Phytochemistry 58, 587-590.

Kitahara, M., Sakamoto, M., Benno, Y., 2001a. PCR detection method of Clostridium scindens and C. hiranonis in human fecal samples. Microbiol. Immunol. 45, 263-266.

Kitahara, M., Takamine, F., Imamura, T., Benno, Y., 2001b. Clostridium hiranonis sp nov., a human intestinal bacterium with bile acid 7 alpha-dehydroxylating activity. Int. J. Syst. Evol. Microbiol. 51, 39-44.

Liggins, J., Grimwood, R., Bingham, S.A., 2000. Extraction and quantification of lignan phytoestrogens in food and human samples. Anal. Biochem. 287, 102-109.

Mazur, W., Fotsis, T., Wähälä, K., Ojala, S., Salakka, A., Adlercreutz, H., 1996. Isotope dilution gas chromatographic mass spectrometric method for the determination of isoflavonoids, coumestrol, and lignans in food samples. Anal. Biochem. 233, 169-180.

Mechichi, T., Labat, M., Patel, B.K.C., Woo, T.H.S., Thomas, P., Garcia, J.L., 1999. Clostridium methoxybenzovorans sp nov., a new aromatic o-demethylating homoacetogen from an olive mill wastewater treatment digester. Int. J. Syst. Bacteriol. 49, 1201-1209.

Muller, J.A., Schink, B., 2000. Initial steps in the fermentation of 3-hydroxybenzoate by Sporotomaculum hydroxybenzoicum. Arch. Microbiol. 173, 288-295.

Muller, V., Imkamp, F., Rauwolf, A., Kusel, K., Drake, H.L., 2004. Molecular and cellular biology of acetogenic bacteria. In: Nakano, M.M., Zuber, P. (Eds.), Strict and facultative anaerobes: Medical and environmental aspects. Horizon Bioscience, Wymondham, pp. 251281.

Nemeth, K., Plumb, G.W., Berrin, J.G., Juge, N., Jacob, R., Naim, H.Y., Williamson, G., Swallow, D.M., Kroon, P.A., 2003. Deglycosylation by small intestinal epithelial cell beta-
glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans. Eur. J. Nutr. 42, 29-42.

Possemiers, S., Heyerick, A., Robbens, V., De Keukeleire, D., Verstraete, W., 2005. Activation of proestrogens from hops (Humulus lupulus L.) by intestinal microbiota; Conversion of isoxanthohumol into 8-prenylnaringenin. J. Agric. Food Chem. 53, 62816288.

Schöttner, M., Gansser, D., Spiteller, G., 1997. Lignans from the roots of Urtica dioica and their metabolites bind to human sex hormone binding globulin (SHBG). Planta Med. 63, 529-532.

Setchell, K.D.R., Lawson, A.M., Borriello, S.P., Harkness, R., Gordon, H., Morgan, D.M.L., Kirk, D.N., Adlercreatz, H., Anderson, L.C., Axelson, M., 1981. Lignan formation in man-microbial involvement and possible roles in relation to cancer. Lancet 2, 4-7.

Setchell, K.D.R., Brown, N.M., Zimmer-Nechemias, L., Brashear, W.T., Wolfe, B.E., Kirschner, A.S., Heubi, J.E., 2002. Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. Am. J. Clin. Nutr. 76, 447-453.

Struijs, K., Vincken, J.-P., Verhoef, R., van Oostveen-van Casteren, W.H.M., Voragen, A.G.J., Gruppen, H., 2007. The flavonoid herbacetin diglucoside as a constituent of the lignan macromolecule from flaxseed hulls. Phytochemistry 68, 1227-1235.

Wang, L.Q., 2002. Mammalian phytoestrogens: enterodiol and enterolactone. J. Chromatogr. B 777, 289-309.

Wang, L.-Q., Meselhy, M.R., Li, Y., Qin, G.-W., Hattori, M., 2000. Human intestinal bacteria capable of transforming secoisolariciresinol diglucoside to mammalian lignans, enterodiol and enterolactone. Chem. Pharm. Bull. 48, 1606-1610.

Yamamoto, S., Otto, A., Simoneit, B.R.T., 2004. Lignans in resin of Araucaria angustifolia by gas chromatography/mass spectrometry. J. Mass Spectrom. 39, 1337-1347.

# Chapter 6 <br> Comparison of APCI-MS and ESI-MS for the detection of lignans from sesame seeds 



Chapter 6


#### Abstract

In sesame seeds, high concentrations of lignans are present. When these lignans are fermented in the human colon, a range of structurally different lignans is formed. A good LC-MS protocol for the analysis of lignans in complex mixtures is lacking. In order to develop such a protocol, ESI-MS and APCI-MS, both in the positive and negative ionization mode, were compared. An extract from defatted sesame meal was analyzed by APCI-MS and ESI-MS, before and after deglucosylation. APCI-MS was found to be a more generic method compared to ESIMS, because especially sesamolin, sesamin and pinoresinol, were better detected by APCIMS than by ESI-MS. Positive and negative ionization modes had to be combined in order to detect all lignans in a bacterial culture grown on aglyconic, acid-treated lignans from sesame oil and defatted sesame meal. Lignans with methylenedioxy-bridged furanofuran structures mostly lack phenolic hydroxyl groups and were, therefore, optimally detected in positive ionization mode. Dibenzylbutadiene lignans, which were formed during fermentation, carry hydroxyl groups and were better detected in negative ionization mode.


## Introduction

Sesame seeds are utilized for direct human consumption and for oil production (Namiki, 2007). In sesame oil ( $45 \% \mathrm{w} / \mathrm{w}$ of the seeds), high concentrations of lignans are present (Moazzami and Kamal-Eldin, 2006), which are held responsible for the oxidative stability of sesame oil (Fukuda et al., 1994). Sesamin and sesamolin were identified as the major lignans in oil, representing $0.4-0.6 \%(\mathrm{w} / \mathrm{w})$ of the oil (Dachtler et al., 2003; Lee and Choe, 2006). In defatted sesame meal mainly sesaminol- and sesamolinol glucosides were identified, accounting for 1.3 \% (w/w) of the defatted meal (Dachtler et al., 2003; Katsuzaki et al., 1994a; Moazzami et al., 2006a; Moazzami et al., 2006b). In Fig. 1 the chemical structures of the major lignans from sesame seeds are shown.
Recently, it was shown that lignans from sesame seeds can be converted into the bioactive mammalian lignan enterodiol (END; see Fig. 1 for chemical structure) in the human body either by direct fermentation (Penalvo et al., 2005) or by fermentation after enterohepatic circulation (Nakai et al., 2003). After absorption by the colon, the methylenedioxy bridges can be cleaved by liver enzymes and various intermediates are formed (Nakai et al., 2003). Via the enterohepatic circulation these intermediates end up in the colon where they can be fermented resulting in the formation of END (Liu et al., 2006; Penalvo et al., 2005). To be able to determine the structurally different intermediates formed during fermentation of lignans from sesame seeds, a suitable analytical protocol is needed covering the identification of all lignans present.

In the literature, often GC-MS is used for the identification of lignans from sesame seeds (Amarowicz et al., 2001; Grace et al., 2003b; Kamal-Eldin et al., 1994; Liu et al., 2006; Penalvo et al., 2005). The disadvantage of GC-MS is that derivatisation is needed to make the non-volatile lignans detectable. LC-MS methods do not have this disadvantage and could, therefore, be a good alternative. LC-MS has proven its usefulness for the analysis and identification of specific lignans and lignan glycosides (Grace et al., 2003a; Kang et al., 2006; Struijs et al., 2008; Wong et al., 2000; Ye et al., 2005). However, there is no literature describing a method for the detection of the full spectrum of lignans from sesame seeds in complex mixtures such as fermentation cultures.
In order to develop a protocol for the detection and identification of a broad spectrum of lignans in complex mixtures, in the present study a comparison was made between two ionization techniques (ESI-MS and APCI-MS) and two ionization modes (positive and negative) in an LC-MS system. Sesame seeds were used as a source of lignans.

sesamin $\mathrm{C}_{20} \mathrm{H}_{18} \mathrm{O}_{6}$ Mw 354.1

pinoresinol (PINO)
$\mathrm{C}_{20} \mathrm{H}_{22} \mathrm{O}_{6}$ Mw 358.1

$\mathrm{R}=\mathrm{H}$ : sesamino
$\mathrm{C}_{20} \mathrm{H}_{18} \mathrm{O}_{7}$
Mw 370.1
Mw 370.1
$\mathrm{R}=x \mathrm{GIc}$ : sesaminol glucoside

$\stackrel{\text { samin }}{\mathrm{C}_{13} \mathrm{H}_{14} \mathrm{O}_{5}}$ Mw 250.1

$\mathrm{R}=\mathrm{H}$ : secoisolariciresinol (SECO) $\mathrm{C}_{20} \mathrm{H}_{26} \mathrm{O}_{6}$
$\mathrm{C}_{20} \mathrm{H}_{26} \mathrm{O}_{6}$
Mw 362.2
R=Glc: secoisolariciresinol diglucoside (SDG)

enterodiol (END)
$\mathrm{C}_{18} \mathrm{H}_{22} \mathrm{O}_{4}$
Mw 302.2

Figure 1. Chemical structures of lignans from sesame seeds, the main lignan from flaxseed (SDG) and one of the mammalian lignans (END). * indicates chiral C-atom.

## Results

## Determination of lignans present in sesame seeds

Extraction of lignans from sesame oil and defatted sesame meal
Sesamin and sesamolin were extracted from sesame oil. The chromatogram of the oil extract showed three major peaks (Fig. 2). The largest ones were expected to be sesamin and sesamolin, since they were reported to be the most abundant lignans in sesame oil (Shyu and Hwang, 2002).
Sesaminol glucosides were extracted from defatted sesame meal. The chromatogram of the defatted sesame meal extract (Fig. 3A), showed a series of peaks (peaks 5-11), of which the largest ones were expected to be the sesaminol glucosides.


Figure 2. RP-HPLC profile of lignans extracted from sesame oil. Peak numbers correspond to assignments given in Table 2.

Determination of optimal settings for ESI-MS and APCI-MS
In order to determine which MS-settings are the most optimal for the detection of lignans from sesame seeds, the above described extracts were analyzed for sesamin, sesamolin and sesaminol glucosides with ESI-MS and APCI-MS, both in positive and negative ionization mode, using several tune settings (see experimental section). The ionization and detection of lignans from sesame seeds were compared with the main lignan present in flaxseed, secoisolarisirecinol diglucoside (SDG).
In Table 1, an overview of the detection of the lignans under the various ionization conditions is given. For sesamin and sesamolin, the corresponding $\mathrm{m} / \mathrm{z}$-ratios were detected in the oil extract. In the defatted sesame meal extract, the $m / z$-ratio of sesaminol diglucoside
$\left(\mathrm{m} / \mathrm{z}=693.0[\mathrm{M}-\mathrm{H}]^{-}\right.$or $\left.717.3[\mathrm{M}+\mathrm{Na}]^{+}\right)$was found to be the most abundant and was, therefore, used for the initial method development.


Figure 3. RP-HPLC profiles of lignans from defatted meal before (A) and after ( $\mathrm{B}+\mathrm{C}$ ) deglucosylation by Rapidase $\mathrm{Liq}^{+}$. A = defatted sesame meal extract, $\mathrm{B}=\mathrm{MeOH}$ extract of the pellet formed during deglucosylation of the defatted sesame meal extract, $\mathrm{C}=$ supernatant obtained after deglucosylation of the defatted sesame meal extract. Numbers correspond to assignments in Table 2.

ESI-MS in positive ionization mode resulted in the detection of SDG and sesaminol diglucoside. The glucosides were detected as sodium-adducts ( $[\mathrm{M}+\mathrm{Na}]^{+}$) or as protonadducts $\left([M+H]^{+}\right)$depending on the tune settings. Also sesamin ( $\mathrm{m} / \mathrm{z}=336.9\left[\mathrm{M}-\mathrm{H}_{2} \mathrm{O}+\mathrm{H}\right]^{+}$) was detected, but only with the tune settings of SDG. Using ESI-MS in the negative ionization mode, both sesamin and sesamolin could not be detected, while the glucosides were detected properly.
Using APCI-MS in positive ionization mode only aglyconic lignans were detected. SDG and sesaminol diglucoside were not detected as such, but $\mathrm{m} / \mathrm{z}$-ratios corresponding to their aglycons could be identified (data not shown).
Using APCI-MS in the negative ionization mode sesamolin, sesaminol diglucoside and SDG were detected. An $m / z$-ratio of 353 ( $[\mathrm{M}-\mathrm{H}]^{-}$) corresponding to sesamin was not found. As far as $\mathrm{MS}^{2}$ fragmentation patterns were available for the major lignans, the patterns found (Table 1) were in accordance with literature (Dachtler et al., 2003; Eklund et al., 2008; Lee and Choe, 2006; Yan et al., 2007).
Subsequently, the tune settings that resulted in the identification of the most lignans (ESIMS in positive and negative ionization mode: tune settings of SDG; APCI-MS in negative ionisation mode: tune settings of SDG; APCI-MS in the positive ionization mode: tune settings of sesamin), were tested for their applicability to identify lignans in the oil and defatted sesame meal extract.

Table 1. Detection of lignans from sesame seeds by ESI-MS and APCI-MS in positive and negative ionization mode compared to SDG, the main lignan from flax seed.

|  | $\begin{aligned} & \text { Sesamin } \\ & (\mathrm{Mw}=354.1) \end{aligned}$ | $\begin{aligned} & \hline \text { Sesamolin } \\ & (\mathrm{Mw}=370.1) \end{aligned}$ | Sesaminol diglucoside $(\mathrm{Mw}=694.2)$ | $\begin{aligned} & \hline \text { SDG } \\ & (\mathrm{Mw}=686.3) \end{aligned}$ |
| :---: | :---: | :---: | :---: | :---: |
| ESI-MS + mode |  |  |  |  |
| Tuned by SDG | $337.0^{\text {a }}$ | ND ${ }^{\text {b }}$ | $717.3^{\text {c }}$ | $709.3{ }^{\text {c }}$ |
|  | MS ${ }^{2}$ :319.1,289.1,267.1 |  | MS ${ }^{2}: 555.1,485.1,347.1$ | MS ${ }^{2}: 547.2$ |
| Tuned by SECO | ND ${ }^{\text {b }}$ | ND ${ }^{\text {b }}$ | $694.8{ }^{\text {d }}$ | $686.7{ }^{\text {d }}$ |
|  |  |  | MS ${ }^{2}$ : 485.1, 346.9 | MS ${ }^{2}$ :ND |
| ESI-MS - mode |  |  |  |  |
| Tuned by SDG | ND ${ }^{\text {e }}$ | ND ${ }^{\text {e }}$ | $693.0{ }^{\text {f }}$ | $685.3{ }^{\text {f }}$ |
|  |  |  | MS ${ }^{2}$ : 587.3, 369.0 | MS ${ }^{2}$ :361.2,523.2 |
| Tuned by SECO | $N D^{\text {e }}$ | ND ${ }^{\text {e }}$ | $692.9{ }^{\text {f }}$ | $685.4^{\text {f }}$ |
|  |  |  | MS ${ }^{2}$ : ND | MS ${ }^{2}$ :523.2,361.2 |
| APCI-MS + mode |  |  |  |  |
| Tuned by SECO | 336.9 ${ }^{\text {a }}$ | ND ${ }^{\text {b }}$ | ND ${ }^{\text {b }}$ | $N D^{\text {b }}$ |
|  | MS ${ }^{2}$ :ND |  |  |  |
| Tuned by sesamin | $336.8{ }^{\text {a }}$ | $352.7{ }^{\text {a }}$ | ND ${ }^{\text {b }}$ | ND ${ }^{\text {b }}$ |
|  | MS ${ }^{2}: 318.9,288.9,266.9$ | MS ${ }^{2}$ :ND |  |  |
| APCI-MS - mode |  |  |  |  |
| Tuned by SDG | $N D^{\text {e }}$ | $368.9{ }^{\text {f }}$ | $693.4{ }^{\text {f }}$ | $685.3{ }^{\text {f }}$ |
|  |  | MS ${ }^{2}: 350.9,338.9,200.8$ | MS ${ }^{2}$ : 632.8, 368.9 | MS ${ }^{2}$ :ND |
| Tuned by SECO | $N D^{e}$ | $368.9{ }^{\text {f }}$ | $693.1{ }^{\text {f }}$ | $685.1{ }^{\text {f }}$ |
|  |  | MS ${ }^{2}: 350.8,338.8,200.9$ | MS ${ }^{2}$ :608.3 | MS ${ }^{2}$ :523.1,361.1 |

ND = not detected
${ }^{\mathrm{a}}\left[\mathrm{M}-\mathrm{H}_{2} \mathrm{O}+\mathrm{H}\right]^{+}$
${ }^{\mathrm{b}}$ no detection of $[\mathrm{M}+\mathrm{H}]^{+},[\mathrm{M}+\mathrm{Na}]^{+},\left[\mathrm{M}-\mathrm{H}_{2} \mathrm{O}+\mathrm{H}\right]^{+}$
${ }^{\text {c }}$ present as sodium adduct
${ }^{\mathrm{d}}[\mathrm{M}+\mathrm{H}]^{+}$
${ }^{e}$ no detection of $[\mathrm{M}-\mathrm{H}]^{-}$or an adduct with acetic acid
${ }^{\mathrm{f}}[\mathrm{M}-\mathrm{H}]^{-}$
Identification of all lignans from sesame seeds by ESI-MS and APCI-MS
In the extracts of the sesame oil and defatted sesame meal several components were present as shown by the peaks of RP-HPLC (Fig. 2 and Fig. 3A). The $m / z$-ratios (ESI-MS and APCI-MS) found at the RT of the peaks in Fig. 2 and 3 are shown in Table 2. By comparisons with control samples, peaks 1-4 were shown to originate from the enzyme mixture.
Based on the MS data (see Table 2), peaks 5-11 (Fig. 3A) were assigned as sesaminol glucosides. The most abundant peak, peak 8, was assigned as sesaminol diglucoside, while the triglucoside was expected to be the most abundant based on literature (Moazzami et al., 2006b; Shyu and Hwang, 2002). Multiple peaks were assigned as sesaminol triglucosides (peaks 5 and 6) and sesaminol diglucosides (peaks 7 and 8), consistent with the occurrence of various isomers of sesaminol (Nagata et al., 1987) and variation in the linkages types of the sugar chains (Katsuzaki et al., 1994b).
Peak 10 was assigned as pinoresinol (PINO) based on the $m / z$-ratio of $340.8\left(\left[\mathrm{M}-\mathrm{H}_{2} \mathrm{O}+\mathrm{H}\right]^{+}\right)$ found by APCI-MS in the positive ionization mode. Although also present in the extract from sesame meal, peaks 16 and 17 originated from sesame oil, and were assigned as sesamin and sesamolin, respectively. ESI-MS and APCI-MS in the negative ionization
mode showed most optimal results for detection of the glucosides. To investigate whether these procedures were also the most optimal for the detection of aglyconic lignans, the defatted sesame meal extract was deglucosylated and analyzed using the same methods as the defatted meal extract.

## Determination of deglucosylated lignans from defatted sesame meal

## Deglucosylation of lignans from sesame meal

In order to obtain the sesaminol aglycon, the sesame meal extract was enzymatically deglucosylated. During enzyme treatment, the deglucosylated components became partially insoluble in water. In order to recover the insoluble components, they were resolubilized from the pellet in MeOH before analysis. The RP-HPLC profiles of the aqueous supernatant and the resolubilized pellet of deglucosylated sesame meal extract are shown in Fig. 3. Compounds 12-17 were partially insoluble in water and were mainly found back in the pellet (Fig. 3B), while others remained soluble and were analyzed in the aqueous supernatant (Fig. 3C). The major peak formed after deglucosylation (peak 14) was expected to be sesaminol.

## Determination of all deglucosylated lignans by ESI-MS and APCI-MS

The three new peaks formed during deglucosylation (peaks 13-15) were all assigned as sesaminol based on the MS and MS ${ }^{2}$ data (Table 2) and were suggested to be different isomers (Nagata et al., 1987). Using ESI in the positive ion mode only traces of sesaminol were found, whereas in the negative ionization mode it was detected properly ( $\mathrm{m} / \mathrm{z}=369.1$ $[\mathrm{M}-\mathrm{H}]^{-}$). Using APCI-MS in the positive ionization mode, both the water eliminated ( $\mathrm{m} / \mathrm{z}=$ $\left.352.8\left[\mathrm{M}-\mathrm{H}_{2} \mathrm{O}+\mathrm{H}\right]^{+}\right)$and the protonated forms $\left(\mathrm{m} / \mathrm{z}=370.7[\mathrm{M}+\mathrm{H}]^{+}\right)$were detected.
The MS ${ }^{2}$ fragmentation patterns found for sesaminol were similar for APCI-MS and ESIMS. Using the positive ionization mode, product ions with $m / z$-ratios of $334.8,322.8,305.0$ and 134.9 were found. When using the negative ionization mode fragments with $\mathrm{m} / \mathrm{z}$-ratios of 350.7, 339.0, 219.0 and 190.9 were identified. So far, only limited data on the fragmentation mechanism of sesaminol have been reported (Eklund et al., 2008). Based on literature (Eklund et al., 2008; Yan et al., 2007; Ye et al., 2005) and fragmentation observed in this study, tentative fragmentation pathways for sesaminol in the negative and positive ionization mode are presented in Fig. 4. In the negative ionization mode, after deprotonation, cleavage through the furan ring structures is suggested to take place (Fig. 4A). This is in analogy with the fragmentation described for PINO (Ye et al., 2005). In the positive mode, water elimination on the furan-ring took place, followed by either a second water elimination or formaldehyde elimination (Fig. 4B).

Besides peaks $13-15$, peak 12 was present in the deglucosylated sesame meal extract. For peak 12, just as for peaks 13-15 and peak 17, depending on the ionization mode, $\mathrm{m} / \mathrm{z}$-ratios of $370.7\left([\mathrm{M}+\mathrm{H}]^{+}\right)$, $352.8\left(\left[\mathrm{M}-\mathrm{H}_{2} \mathrm{O}+\mathrm{H}\right]^{+}\right)$, or $368.9\left([\mathrm{M}-\mathrm{H}]^{-}\right)$were found. They all corresponded to a molecular mass of 370 Da. Peaks 13-15 were assigned as sesaminol and peak 17 was assigned as sesamolin because it had the same RT as the compound in the sesame oil extract. Peak 12 showed a different ionization and fragmentation behavior compared to peaks 13-15 and 17 . For peak 12 mainly a protonated molecule $\left([\mathrm{M}+\mathrm{H}]^{+}\right)$was


Figure 4. Proposed fragmentation of sesaminol. (A) in the negative ionization mode. (B) in the positive ionization mode.

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found, while for the other peaks the water eliminated molecule ( $\left[\mathrm{M}-\mathrm{H}_{2} \mathrm{O}+\mathrm{H}\right]^{+}$) was dominant. Also the $\mathrm{MS}^{2}$ fragmentation in positive ionisation mode of peak 12 ( $\mathrm{MS}^{2}$ : m/zratios $352.9,340.6,230.6$ ) differed from the fragmentation of the other peaks ( $\mathrm{MS}^{2}: m / \mathrm{z}$ ratios $334.8,322.8,305.0,134.9$ ), showing that peak 12 should not be assigned as sesaminol (peak 13, 14 and 15) or sesamolin (peak 17). Besides sesaminol and sesamolin, epi-sesaminone has previously been identified in sesame seeds as one of the components with a molecular mass of 370 Da (Marchand et al., 1997). Therefore, peak 12 was suggested to correspond to epi-sesaminone.
Sesamin, sesamolin, epi-sesaminone and PINO were only detected by APCI-MS. This, together with the observation that the other lignans are also detected by APCI-MS, showed that APCI-MS was best suited for the identification of aglyconic lignans from sesame seeds in comparison to ESI-MS. The APCI-MS protocol was validated by analyzing lignans in fermentation cultures.

## Determination of lignans in fermentation cultures

It has been suggested that before any fermentation of lignans from sesame seeds can occur, the methylenedioxy bridges should be cleaved (Liu et al., 2006; Nakai et al., 2003; Penalvo et al., 2005). To chemically achieve the opening of the methylenedioxy bridges (or diepoxides), the lignans were acid treated as this was expected to result in ring opening of epoxides (Allinger et al., 1971). The acid concentrations used were optimized (results not shown), compromising between the formation and loss of compounds of interest.
In Table 3, the $m / z$-values found at the various retention times on RP-HPLC (chromatogram not shown) of the acid pretreated and fermented lignans are given, together with the $\mathrm{m} / \mathrm{z}$ ratios of the $\mathrm{MS}^{2}$ fragments and their assignments. The peak with RT 7.1 min could not be identified. The peaks with RT 17.9, 21.6 and 23.2 min were assigned as PINO based on $\mathrm{m} / \mathrm{z}$-values found in positive ionization mode ( $\mathrm{m} / \mathrm{z}=340.8\left[\mathrm{M}-\mathrm{H}_{2} \mathrm{O}+\mathrm{H}\right]^{+}$). The peaks with RT 17.9 and 23.2 min were formed during acid treatment. The formation of these two new peaks of PINO can be explained by an isomerisation reaction of naturally present PINO (RT 21.6 min ) under influence of acid, as described previously for sesamin (Li et al., 2005).

The compound at RT 26.9 min was formed upon acid treatment and was assigned as samin $\left(\mathrm{m} / \mathrm{z}=232.6[\mathrm{M}+\mathrm{H}]^{+}\right)$, based on the results of APCI-MS in positive ionization mode. Samin was hypothesized to be formed out of sesamolin during the acid treatment (Fukuda et al., 1986; Fukuda et al., 1994). At the RT of sesamolin (Table 2), no peak was observed anymore after acid treatment (chromatogram not shown).
For the peaks with RT 29.6, 32.8 and 34.6 min , no $\mathrm{m} / \mathrm{z}$-ratios were found at the beginning of the fermentation, while after 24 h they were detected. The RT and the $\mathrm{m} / \mathrm{z}$-ratios of these peaks corresponded to the RT and $m / z$-ratios of peaks 12,14 and 15 in Table 2, and were assigned accordingly. Peak 13 (Table 2), which was also assigned as sesaminol in the deglucosylated sesame meal extract, was not present any more after acid treatment. Therefore, it is suggested that acid treatment resulted in the two most stable isoforms of sesaminol (sesaminol and 6-episesaminol) (Nagata et al., 1987).
Sesamin itself was not affected by the acid treatment, probably due to the low acid concentration. It was concluded that the acid treatment was not effective in cleaving the methylenedioxy bridges. Nevertheless, the acid treatment resulted in several new compounds of which the fermentability was investigated.

| peak | $\begin{aligned} & \hline \text { RT } \\ & (\mathrm{min}) \end{aligned}$ | In sample |  | ESI-MS ${ }^{\text {a }}$ |  | APCI-MS ${ }^{\text {a }}$ |  | MS ${ }^{2}$ |  | Assignment |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  | -liq ${ }^{+}$ | $+\mathrm{liq}^{+}$ | + mode | - mode | + mode | - mode | + mode | - mode |  |
| 5 | 15.2 | + | ND ${ }^{\text {b }}$ | $879.3{ }^{\text {c }}$ | $\begin{aligned} & \hline 855.2 \\ & 914.8^{\mathrm{d}} \end{aligned}$ | ${ }^{\text {e }}$ | - | 717.3,527.2,305.1 | 705.0,693.3,484.8 | Sesaminol triglucoside |
| 6 | 16.4 | + | + | $897.3^{\text {c }}$ | 855.2 | $879.1^{\text {c }}$ | 914.6 ${ }^{\text {d }}$ | 717.3,647.2,509.1 | 693.0,485.0,369.1 | Sesaminol triglucoside |
| 7 | 17.9 | + | + | $717.3{ }^{\text {c }}$ | 693.1 |  | 692.9 | 555.2,365.1,244.9 | - | Sesaminol diglucoside |
| 8 | 19.5 | + | - | $717.3{ }^{\text {c }}$ | 692.9 | $\begin{aligned} & 716.8^{f} \\ & 694.4^{f} \end{aligned}$ | $\begin{aligned} & 693.8 \\ & 368.9 \end{aligned}$ | 555.2,485.1,347.0 | 519.2,262.7 | Sesaminol diglucoside |
| 9 | 20.0 | + | + | 717.2 | 693.1 | 368.8 | 693.3 | 555.2,485.1,347.1 | 369.0 | Sesaminol diglucoside |
| 10 | 21.7 | + | + | - | 371.2 | $340.8^{\text {g }}$ | - | 322.9,290.9,270.9 | - | Pinoresinol |
| 11 | 23.3 | + | + | 555.2 | 591.0 | - | $\begin{aligned} & 590.7^{\mathrm{d}} \\ & 368.9 \end{aligned}$ | 537.0,323.0,184.8 | $\begin{aligned} & \text { 564.8,509.5,368.9 } \\ & 339.0,218.8,190.9 \end{aligned}$ | Sesaminol monoglucoside |
| 12 | 29.8 | ND ${ }^{\text {b }}$ | + | - | - | 370.7 | - | 352.8,340.9,230.6 | -0,218.8,190.9 | Epi-sesaminone ${ }^{\text {h }}$ |
| 13 | 32.2 | ND ${ }^{\text {b }}$ | + | $353.0^{\text {g }}$ | 369.1 | $\begin{aligned} & 352.8^{\mathrm{g}} \\ & 370.7 \end{aligned}$ | - | 334.8,322.8,304.7 | 322.9,135.1 | Sesaminol |
| 14 | 33.0 | ND ${ }^{\text {b }}$ | + | - | 369.1 | $\begin{aligned} & 352.8^{\mathrm{g}} \\ & 370.7 \end{aligned}$ | 368.9 | 334.8,304.9,134.9 | 339.1,219.0,191.0 | Sesaminol |
| 15 | 34.5 | ND ${ }^{\text {b }}$ | + | - | 369.1 | $352.8{ }^{\text {g }}$ | 368.9 | 334.9,322.7,305.0 | 338.9,219.9,190.9 | Sesaminol |
| 16 | 39.2 | + | + | - | - | $336.8^{\text {g }}$ | - | 318.8,288.9,135.1 | - | Sesamin |
| 17 | 44.4 | + | + | - | - | - | 368.8 | - | 350.7,338.8,219.1 | Sesamolin |

[^0]Table 3. Detection of lignans by RP-HPLC-APCI-MS in the positive ( $[\mathrm{M}+\mathrm{H}]^{+}$or $\left[\mathrm{M}-\mathrm{H}_{2} \mathrm{O}+\mathrm{H}\right]^{+}$) and negative ( $[\mathrm{M}-\mathrm{H}]^{-}$) ionization mode during fermentation of aglyconic acid treated lignans from sesame oil and meal.

| $\begin{aligned} & \mathrm{RT} \\ & (\mathrm{~min}) \end{aligned}$ | Positive ionization mode |  |  | Negative ionization mode |  |  | $\mathrm{MS}^{2}$ |  | Assignment |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | t0 ${ }^{\text {a }}$ | t24 | t96 | t0 | t24 | t96 | + mode | - mode |  |
| 7.1 | 196.9 | 196.9 | 196.9 | $318.6{ }^{\text {d }}$ | 253.0 | 318.5 | 168.8,140.9,123.9 | - | Unidentified |
|  |  | 261.9 | 260.9 |  |  |  | 232.8,154.9,135.9 | - |  |
| 17.0 | ND ${ }^{\text {b }}$ | - ${ }^{\text {c }}$ | ND | ND | 361.1 | ND | 23.8,154.9,135.9 | 345.8,330.8,164.9 | SECO |
| 17.9 | 340.8 | 340.8 | 340.7 | - | - | $371.0^{\text {de }}$ | 322.9,290.9,270.9 | 34,8,330.8,164.9 | Pinoresinol ${ }^{\text {f }}$ |
| 20.0 | ND | ND | - | ND | ND | 301.1 | 32.9,290.9,270.9 | 283.0,271.2,253.1 | Enterodiol |
| 21.6 | 340.8 | ND | ND | $369.1{ }^{\text {e }}$ | ND | ND | 322.9,290.9,271.0 | 290.7,263.0 | Pinoresinol ${ }^{\text {f }}$ |
| 23.2 | ND | ND | - | ND | ND | - | - | - | Unidentified |
| 23.2 | 340.8 | 340.7 | ND | $368.9{ }^{\text {e }}$ | - | ND | 323.0,290.8,271.0 | - | Pinoresinol ${ }^{\text {f }}$ |
|  |  |  |  | 590.9 |  |  | 323.0,290.8,271.0 | - |  |
| 26.9 | 232.7 | 232.6 | 232.6 | - | - | - | 215.0,203.6,119.8 | - | Samin |
| 29.6 | - | 370.6 | 370.7 | - | - | - | 352.9,337.7,340.5 | - | Epi-sesaminone ${ }^{\text {g }}$ |
| 32.8 | - | 352.8 | 352.8 | 368.8 | 368.9 | 368.9 | 334.8,304.9,135.0 | 350.7,338.9,219.1 | Sesaminol |
| 34.6 | - | 352.8 | 352.8 | 368.8 | 368.9 | 368.9 | 335.1,304.8,135.1 | 351.0,338.7,218.7 | Sesaminol |
| 39.0 | 336.8 | 336.8 | 336.8 | 333.6 | 326.0 | - | 318.9,289.0,134.9 |  | Sesamin |

[^1]PINO with RT 21.6 min was converted within the first 24 h of fermentation, followed by PINO with RT 23.2 min in the next 72 h . PINO with RT 17.9 min was not fermented at all. This suggested that the configuration of molecules influences the rate of fermentation (Jin et al., 2007).
After 24 h of fermentation, a compound with RT 17.0 min and $\mathrm{m} / \mathrm{z}$-ratio of 361.1 ([M-H] ${ }^{-}$) was formed. This compound was identified as secoisolariciresinol (SECO) based on the MS and $\mathrm{MS}^{2}$ data (Table 3). After 96 h of incubation, a compound, which elutes at 20.0 min was formed. This compound had an $\mathrm{m} / \mathrm{z}$-ratio of 301.1 ( $[\mathrm{M}-\mathrm{H}]^{-}$) and was assigned as END. Also a compound, which eluted at 23.2 min was formed, but this compound could not be identified. For SECO and END, $m / z$-ratios were found only in negative ionization mode. These results showed that PINO, samin, sesaminol and sesamin (the furanofuran lignans) were best detected in positive ionisation mode while SECO and END (the dibenzylbutadiene lignans) were best detected in negative ionisation mode.

## Discussion

## Comparison of APCI-MS and ESI-MS

APCI-MS was found to be the ionization technique by which most lignans from sesame seeds could be detected. In the case of ESI-MS, dibenzylbutadiene lignans were detected properly (Eeckhaut et al., 2008; Struijs et al., 2008), while only some of the furanofuran lignans were detected. The difference in ionization mechanism between APCI-MS and ESIMS might explain these differences. Of the two mechanisms, proton transfer is dominant for APCI-MS, while for ESI-MS droplet disintegration is predominant (Niessen, 2006).
The surface activity of a molecule determines its chances of ionization during droplet disintegration. Surface-active molecules reside at the edge of a droplet and are ionized during droplet disintegration, while less surface-active molecules are less likely ionized (Cech and Enke, 2001). Most furanofuran lignans have a relatively ridged, almost symmetrical structure, which makes them poorly surface-active. Therefore, they are poorly ionized and detected by ESI-MS.
Sesamolin can not be detected by ESI-MS (under the conditions used), while it is detected by APCI-MS. For APCI-MS, proton transfer plays an important role. Apparently, the proton transfer is efficient between sesamolin and ions formed by corona discharge in case of APCI-MS. For ESI-MS, the proton transfer potential between ions formed during droplet disintegration and sesamolin was not high enough to ionize sesamolin.
External solvent ions, with an expected high proton transfer potential, being responsible for the ionization, explains that APCI-MS is a more universally applicable ionization method compared to ESI-MS. Nevertheless, ESI-MS is used often, because it is a more sensitive method (de Rijke et al., 2003; Grace et al., 2003a; Niessen, 2006; Rybak et al., 2008).

## Use of positive and negative ionization modes

In line with literature results (Dachtler et al., 2003; Eklund et al., 2008; Lee and Choe, 2006; Yan et al., 2007; Ye et al., 2005) this study showed only detection of sesamin in positive ionzation mode. Apparently, sesamin can not be deprotonated, which is expected since sesamin lacks phenolic hydroxyl groups or other groups with easily leaving protons. SECO and END (dibenzylbutanediol lignans) were better detected in the negative ionisatation mode than in the positive ionisation mode. They are assumed to have a higher proton donative capacity compared to the methylenedioxy-bridged furanofuran lignans (e.g. sesamin) because of their phenolic and non-phenolic hydroxyl groups.
More generally, it can be stated that compounds lacking phenolic hydroxyl groups can not be deprotonated, and are difficult to analyze in the negative ionization mode (Eklund et al., 2008; Ye et al., 2005). Sesamolin was found to be an exception to that. It does not contain a phenolic hydroxyl group, but it was detected by APCI-MS in the negative ionization mode. It is hypothesized that the ether moiety is sufficiently electro negative, which makes the proton at the chiral C-atom next to the ether moiety an easily leaving proton.

In accordance with literature on flavonoid glycosides (Rauha et al., 2001), glucosidic lignans were hard to detect by APCI-MS in the positive mode. It was suggested that the capillary temperature was not high enough to evaporate the glucosides (Rauha et al., 2001)
and that, therefore, they were not detected. A second explanation is that the glucosidic linkage is relative weak and that this linkage is already broken during ionization (Ye et al., 2005). The observed $m / z$-ratio of the aglycon at the RT of the sesaminol glucosides favours the second explanation.

In conclusion, APCI-MS was found to be a more generally applicable ionization method compared to ESI-MS for the detection of lignans. With this method, combining the positive and negative ionization mode, detection of all lignans derived from sesame seeds in complex mixtures, is possible. Methylenedioxy-bridged furanofuran lignans are more easily detected in positive ionization mode, while dibenzylbutanediol type lignans can only be detected in negative ionization mode.

## Experimental

## Extraction of lignans from sesame seed

Sesame seeds were milled prior to soxhlet defatting with hexane ( 0.3 g meal/ ml hexane). After evaporation of the hexane, sesame oil was obtained.
In order to extract the lignans from the oil, oil was extracted with methanol ( $0.2 \mathrm{~g} \mathrm{oil} / \mathrm{ml}$ MeOH ) at $90^{\circ} \mathrm{C}$ for 30 min . The oil was solidified at $-20^{\circ} \mathrm{C}$ and centrifuged to separate the methanolic extract from the oil. Water was added till $40 \%(\mathrm{v} / \mathrm{v})$ to the methanolic extract and residual oil was removed by liquid -liquid extraction with hexane (1:1 (v/v)). After lyophilisation, an oil-like extract was obtained.
Lignans were extracted from the defatted meal with $63 \%$ (v/v) EtOH ( 0.15 g defatted $\mathrm{meal} / \mathrm{ml} 63 \%(\mathrm{v} / \mathrm{v}) \mathrm{EtOH}$ ) twice for 4 hours while stirring at room temperature. EtOH was evaporated and a defatted sesame meal extract was obtained after lyophilization.

## Deglucosylation of glycosidic lignans

Glycosidic lignans were deglycosylated by a crude commercial enzyme extract Rapidase $\mathrm{Liq}^{+}$(DSM, mixture of enzymes from Trichoderma longibrachiatum and Aspergillus niger). $20 \mu$ l Rapidase $\mathrm{Liq}^{+}$was added per ml defatted sesame meal extract ( $2 \mathrm{mg} / \mathrm{ml}$ in 50 mM sodium acetate buffer pH 5 ). The mixture was incubated for 24 hours at $35^{\circ} \mathrm{C}$ while stirring. The reaction was stopped by boiling the solution for 30 min . The supernatant and the pellet were separated by centrifugation ( $30 \mathrm{~min}, 28100 \mathrm{~g}, 4^{\circ} \mathrm{C}$ ).
Lignans were extracted from the supernatant by liquid-liquid partitioning with ethyl acetate (EA) (1:3 (v/v), supernatant: EA). EA was evaporated and the samples were lyophilized.
The pellet was extracted with MeOH in order to obtain the water insoluble lignans. The pellet of 100 ml enzyme-solution (containing 200 mg lignan extract and 2 ml Rapidase $\mathrm{Liq}^{+}$) was extracted with 20 ml MeOH . Pellet and extract were separated by centrifugation (30 min, $28100 \mathrm{~g}, 4^{\circ} \mathrm{C}$ ).

## Fermentation of acid pretreated aglyconic lignans from sesame seeds

The lignan extract from oil and the deglucosylated lignans from defatted meal were acid treated prior to fermentation. 5600 mg lignans from oil were solubilized in 60 ml 0.5 M methanolic HCl , while for the deglucosylated lignans 370 mg was treated with 60 ml 0.05 M methanolic HCl . After 1 hour incubation at $85^{\circ} \mathrm{C}$, the reaction was stopped by neutralizing the samples with NaOH while cooling on ice. The treated lignans from oil and the treated deglucosylated lignans were combined, MeOH was evaporated and the acid treated lignan mixture was redissolved in $20 \mathrm{ml} 20 \%(\mathrm{v} / \mathrm{v})$ aqueous MeOH .
Fermentation media were prepared containing 40 ml brain heart infusion (BHI)-media (40 $\mathrm{g} / \mathrm{L}$ ) plus 5 ml acid treated lignan mixture. Blanks were prepared accordingly. All media were flushed with nitrogen. To these media 5 ml fecal slurry was added. This fecal slurry consisted of 5 gram of feces of 5 healthy volunteers each diluted to a $5 \%(\mathrm{w} / \mathrm{v})$ solution, filtered and made anaerobic by flushing with nitrogen.
The inoculations were incubated at $37^{\circ} \mathrm{C}$. At several time points samples ( 1 ml ) were taken. Lignans were extracted by liquid-liquid partitioning with EA ( $1: 3 \mathrm{v} / \mathrm{v}$ ). After evaporation of the EA, the samples were redissolved in 0.5 ml MeOH and analyzed on RP-HPLC-MS.

## Analysis of the lignan extracts by RP-HPLC

Aglyconic lignans were, prior to RP-HPLC analysis, treated with liquid-liquid partitioning with ethyl acetate and resolubilized in MeOH , as mentioned above. Glycosidic lignans were cleaned by SPE. SepPak Vac C18 cartridges ( $20 \mathrm{cc} / 5 \mathrm{~g}$, Waters) were activated with successively two column volumes MeOH and 2 column volumes water. A sample of lignan glucosides ( $\sim 2 \mathrm{mg} / \mathrm{ml}$ ) in water was loaded and the cartridge was washed with one column volume of water. Lignan glycosides were eluted by 3 column volumes of MeOH of which the first one was discarded. The MeOH was evaporated till a final volume of 1 ml .
Samples were analyzed on a Thermo separation products HPLC system equipped with a membrane degasser, P4000 pump, AS3000 autosampler, and a UV3000 detector. $20 \mu \mathrm{l}$ samples were injected on a Waters XTerra C18 MS column ( $4.6 \times 150 \mathrm{~mm}, 3.5 \mu \mathrm{~m}$ particles size) with guard column (XTerra C18 MS, $4.6 \times 10 \mathrm{~mm}, 3.5 \mu \mathrm{~m}$ particle size, Waters). Water and acetonitrile (ACN), both acidified with $0.1 \%(\mathrm{v} / \mathrm{v})$ acetic acid were used as eluents. The flow rate was $0.7 \mathrm{ml} / \mathrm{min}$, and the eluate was monitored at 280 nm . The flowing gradient was used: 0-30 min, linear from 10\%-50\% (v/v) ACN; 30-45 min, linear from $50 \%-100 \%$ ACN; 45-50 min, isocratic on $100 \%$ ACN; 50-55 min, linear from $100 \%$ $10 \%$ ACN; 55-60 min, isocratic on 10\% ACN.

## Electro Spray Ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI) mass spectrometry (MS)

Mass spectrometrical data were measured on a Thermo Finnigan LCQ Classic coupled on line to the RP-HPLC (see above) equipped with and ESI-MS or APCI-MS probe. In case of ESI-MS, $1 / 10^{\text {th }}$ of the flow from the RP-HPLC was directed to the MS. In case of APCIMS, the total flow was used. Helium was used as sheath gas and nitrogen as auxiliary gas. Data were collected over an $\mathrm{m} / \mathrm{z}$-range of $150-2000$. Data dependent $\mathrm{MS}^{2}$ analysis was performed with a normalized collision energy of $27 \%$. Most settings were optimized using "tune plus" (Xcalibur, Thermo Finnigan) via the automatic and semi-automatic tuning. The capillary temperature, source heater temperature (only APCI), and source voltage were manually adjusted.
For ESI-MS, the system was tuned with SDG and SECO, the SDG aglycon, both in the positive and negative ionization mode (SDG positive: capillary temperature $=270{ }^{\circ} \mathrm{C}$, source voltage $=5 \mathrm{kV}$; SDG negative: capillary temperature $=270^{\circ} \mathrm{C}$, source voltage $=4.5$ kV ; SECO positive: capillary temperature $=150{ }^{\circ} \mathrm{C}$, source voltage $=6.0 \mathrm{kV}$ : SECO negative: capillary temperature $=200^{\circ} \mathrm{C}$, source voltage $=4.5 \mathrm{kV}$ ).
For APCI-MS, the system was tuned with SDG and SECO in the negative ionization mode, and SECO, sesamin and sesamolin in the positive ionization mode. (In all cases, source voltage was 5 kV ; SDG negative: source heater temperature $=450{ }^{\circ} \mathrm{C}$, capillary temperature $=200^{\circ} \mathrm{C}$; SECO negative: source heater temperature $=450^{\circ} \mathrm{C}$, capillary temperature $=200$ ${ }^{\circ} \mathrm{C}$ SECO positive: source heater temperature $=200^{\circ} \mathrm{C}$, capillary temperature $=150^{\circ} \mathrm{C}$; sesamin positive: source heater temperature $=150^{\circ} \mathrm{C}$, capillary temperature $=250^{\circ} \mathrm{C}$ ).
For comparison, all samples were analyzed using four optimized methods for ESI-MS and five optimized methods for APCI-MS.

## References

Amarowicz, R., Shahidi, F., Pegg, R.B., 2001. Application of semipreparative RP-18 HPLC for the purification of sesamin and sesamolin. J. Food Lipids 8, 85-94.

Cech, N.B., Enke, C.G., 2001. Practical implications of some recent studies in electrospray ionization fundamentals. Mass Spectrom. Rev. 20, 362-387.

Dachtler, M., van de Put, F.H.M., von Stijn, F., Beindorff, C.M., Fritsche, J., 2003. On-line LC-NMR-MS characterization of sesame oil extracts and assessment of their antioxidant activity. Eur. J. Lipid Sci. Technol. 105, 488-496.
de Rijke, E., Zappey, H., Ariese, F., Gooijer, C., Brinkman, U.A.T., 2003. Liquid chromatography with atmospheric pressure chemical ionization and electrospray ionization mass spectrometry of flavonoids with triple-quadrupole and ion-trap instruments. J. Chromatogr. A 984, 45-58.

Eeckhaut, E., Struijs, K., Possemiers, S., Vincken, J.-P., De Keukeleire, D., Verstraete, W., 2008. Metabolism of the lignan macromolecule into enterolignans in the gastrointestinal lumen as determined in the simulator of the human intestinal microbial ecosystem. J. Agric. Food Chem. 56, 4806-4812.

Eklund, P.C., Backman, M.J., Kronberg, L.A., Smeds, A.I., Sjoholm, R.E., 2008. Identification of lignans by liquid chromatography-electrospray ionization ion-trap mass spectrometry. J. Mass Spectrom. 43, 97-107.

Fukuda, Y., Isobe, M., Nagata, M., Osawa, T., Namiki, M., 1986. Acidic transformation of sesamolin, the sesame-oil constituent, into an antioxidant bisepoxylignan, sesaminol. Heterocycles 24, 923-926.

Fukuda, Y., Osawa, T., Kawakishi, S., Namiki, M., 1994. Chemistry of lignan antioxidants in sesame seed and oil. In: Ho, C.T., Osawa, T., Huang, M.T., Rosen, R.T. (Eds.), Food phytochemicals for cancer prevention II: Teas, spices and herbs. American chemical society, Washington, pp. 264-274.

Grace, P.B., Taylor, J.I., Botting, N.P., Fryatt, T., Oldfield, M.F., Al-Maharik, N., Bingham, S.A., 2003a. Quantification of isoflavones and lignans in serum using isotope dilution liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass Spectrom. 17, 1350-1357.

Grace, P.B., Taylor, J.I., Botting, N.P., Fryatt, T., Oldfield, M.F., Bingham, S.A., 2003b. Quantification of isoflavones and lignans in urine using gas chromatography/mass spectrometry. Anal. Biochem. 315, 114-121.

Jin, J.S., Zhao, Y.F., Nakamura, N., Akao, T., Kakiuchi, N., Min, B.S., Hattori, M., 2007. Enantioselective dehydroxylation of enterodiol and enterolactone precursors by human intestinal bacteria. Biol. Pharm. Bull. 30, 2113-2119.

Kamal-Eldin, A., Appelqvist, L.Å., Yousif, G., 1994. Lignan analysis in seed oils from four Sesamum species: comparison of different chromatographic methods. J. Am. Oil Chem. Soc. 71, 141-147.

Kang, J., Price, W.E., Hick, L.A., 2006. Simultaneous determination of isoflavones and lignans at trace levels in natural waters and wastewater samples using liquid chromatography/electrospray ionization ion trap mass spectrometry. Rapid Commun. Mass Spectrom. 20, 2411-2418.

Katsuzaki, H., Kawakishi, S., Osawa, T., 1994a. Sesaminol glucosides in sesame seeds. Phytochemistry 35, 773-776.

Katsuzaki, H., Osawa, T., Kawakishi, S., 1994b. Chemistry and antioxidative activity of lignan glucosides in sesame seed. In: Ho, C.T., Osawa, T., Huang, M.T., Rosen, R.T. (Eds.), Food phytochemicals for cancer prevention II: Teas, spices and herbs. American chemical society, Washington, pp. 275-280.

Lee, J.Y., Choe, E., 2006. Extraction of lignan compounds from roasted sesame oil and their effects on the autoxidation of methyl linoleatee. J. Food Sci. 71, C430-C436.

Li, C.Y., Chow, T.J., Wu, T.S., 2005. The epimerization of sesamin and asarinin. J. Nat. Prod. 68, 1622-1624.

Liu, Z., Saarinen, N.M., Thompson, L.U., 2006. Sesamin is one of the major precursors of mammalian lignans in sesame seed (Sesamum indicum) as observed in vitro and in rats. J. Nutr. 136, 906-912.

Marchand, P.A., Kato, M.J., Lewis, N.G., 1997. (+)-episesaminone, a Sesamum indicum furofuran lignan. Isolation and hemisynthesis. J. Nat. Prod. 60, 1189-1192.

Moazzami, A.A., Andersson, R.E., Kamal-Eldin, A., 2006a. Characterization and analysis of sesamolinol diglucoside in sesame seeds. Biosci. Biotechnol. Biochem. 70, 1478-1481.

Moazzami, A.A., Andersson, R.E., Kamal-Eldin, A., 2006b. HPLC analysis of sesaminol glucosides in sesame seeds. J. Agric. Food Chem. 54, 633-638.

Moazzami, A.A., Kamal-Eldin, A., 2006. Sesame seed is a rich source of dietary lignans. J. Am. Oil Chem. Soc. 83, 719-723.

Nagata, M., Osawa, T., Namiki, M., Fukuda, Y., Ozaki, T., 1987. Stereochemical structures of antioxidative bisepoxylignans, sesaminol and its isomers, transformed from sesamolin. Agric. Biol. Chem. 51, 1285-1289.

Nakai, M., Harada, M., Nakahara, K., Akimoto, K., Shibata, H., Miki, W., Kiso, Y., 2003. Novel antioxidative metabolites in rat liver with ingested sesamin. J. Agric. Food Chem. 51, 1666-1670.

Namiki, M., 2007. Nutraceutical functions of sesame: A review. Crit. Rev. Food Sci. Nutr. 47, 651-673.

Niessen, W.M.A., 2006. Atmospheric-pressure ionisation. In: Niessen, W.M.A. (Eds.), Liquid chromatography-mass spectrometry. CRC Press, Boca Raton, pp. 141-176.

Penalvo, J.L., Heinonen, S.M., Aura, A.M., Adlercreutz, H., 2005. Dietary sesamin is converted to enterolactone in humans. J. Nutr. 135, 1056-1062.

Rauha, J.P., Vuorela, H., Kostiainen, R., 2001. Effect of eluent on the ionization efficiency of flavonoids by ion spray, atmospheric pressure chemical ionization, and atmospheric pressure photoionization mass spectrometry. J. Mass Spectrom. 36, 1269-1280.

Rybak, M.E., Parker, D.L., Pfeiffer, C.M., 2008. Determination of urinary phytoestrogens by HPLC-MS/MS: A comparison of atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI). J. Chromatogr. B 861, 145-150.

Shyu, Y.S., Hwang, L.S., 2002. Antioxidative activity of the crude extract of lignan glycosides from unroasted Burma black sesame meal. Food Res. Int. 35, 357-365.

Struijs, K., Vincken, J.-P., Verhoef, R., Voragen, A.G.J., Gruppen, H., 2008. Hydroxycinnamic acids are ester-linked directly to glucosyl moeities within the lignan macromolecule from flaxseed hulls. Phytochemistry 69, 1250-1260.

Wong, S.K., Tsui, S.K., Kwan, S.Y., Su, X.L., Lin, R.C., 2000. Identification and characterization of Podophyllum emodi by API-LC/MS/MS. J. Mass Spectrom. 35, 12461251.

Yan, G.Q., Li, Q.R., Tan, H.R., Ge, T.F., 2007. Electrospray ionization ion-trap time-offlight tandem mass spectrometry of two furofurans: sesamin and gmelinol. Rapid Commun. Mass Spectrom. 21, 3613-3620.

Ye, M., Yan, Y.N., Guo, D.A., 2005. Characterization of phenolic compounds in the Chinese herbal drug Tu-Si-Zi by liquid chromatography coupled to electrospray ionization mass spectrometry. Rapid Commun. Mass Spectrom. 19, 1469-1484.

Chapter 7
General discussion


Chapter 7

In this thesis a detailed description of the lignan macromolecule from flaxseed is given with regard to its subunit composition and structure. Furthermore, the conversion of the main lignan from flaxseeds, secoisolariciresinol (SECO) and its dehydrated form, anhydrosecoisolariciresinol (AHS), into a range of structurally divers lignans by single bacterial strains is described. For the (fermented) lignans from sesame seeds, a method has been developed in which APCI-MS in the positive and the negative ionization mode were combined for the identification of all lignans in a complex extract.

## The structure of the lignan macromolecule

When this research started, it was known that secoisolariciresinol diglucoside (SDG), $p$ coumaric acid glucoside (CouAG) and ferulic acid glucoside (FeAG) were constituents of the oligomeric lignan macromolecule from flaxseeds. Based on the results described in chapter 2 , it is concluded that also the flavonoid herbacetin diglucoside (HDG) is one of the major constituents of the lignan macromolecule.
Until now, flaxseed is the only known source, in which the lignans are linked within an macromolecular structure, although for rye, for example, lignans have been suggested to be linked to cell wall constituents (Smeds et al., 2007). It is currently unknown why lignans in flaxseeds are linked within the lignan macromolecule. Possibly, polymerization allows the plant to store the relatively hydrophobic lignans in the less oily, outer layer of the seed (Madhusudhan et al., 2000).

In Fig. 1 a schematic structure of a lignan macromolecule is presented showing the constituents and how they are linked together. HDG was found to be linked within the lignan macromolecule via 3-hydroxy-3-methyl-glutaric acid (HMGA) moieties similar to SDG (chapter 2). From chapter 3 it can be concluded that CouAG and FeAG are esterlinked to the lignan macromolecule via their carboxylic moiety. CouAG was identified to link to the C-6 position of one of the glucosyl moieties of SDG. For FeAG it remained to be identified how it is actually linked. Ferulic acid ( Fe A ) was found to be linked within the lignan macromolecule via attachment at the C-2 position of a glucosyl moiety of SDG.
As depicted in Fig. 1, another important conclusion of this work is that the hydroxycinnamic acid glucosides (CouAG and FeAG) are present at the terminal positions of the lignan macromolecule. The incorporation of these hydroxycinnmic acids is suggested to terminate the biosynthesis of the lignan macromolecule. An average lignan macromolecule was found to consist of 3 SDG moieties with 2 hydroxycinnamic acid residues on its terminal positions.

Although a fragment of SDG+FeA was identified, FeA was not detected in an extract of fully saponified lignan macromolecule. Therefore, it was regarded as a minor constituent. This is confirmed by a recent publication in which an FeA-ester was identified to contribute for $2 \%$ (w/w) to the lignan macromolecule (Li et al., 2008).
The results on how HDG, CouAG and FeA are linked within the lignan macromolecule could only be obtained by the analysis of fragments of the lignan macromolecule. These
fragments were obtained by partial saponification. Only part of the ester linkages are hydrolyzed, which results in fragments of the lignan macromolecule, which were subsequently identified. It can be concluded that partial saponification is an essential tool for the unraveling of the structure of the lignan macromolecule (Fig. 1).


Figure 1. Chemical structure of a representative of the lignan macromolecule from flaxseed hulls. The gray parts are putative; FeAG might be linked via C-6 or C-2 of a glucosyl moiety of SDG and the quantities of HDG and FeA are low. HMGA and SDG can also be terminal units.

SDG and HDG are linked within the lignan macromolecule via HMGA. During biosynthesis HMGA is coupled to SDG via CoA-activated HMGA (Ford et al., 2001). In the lignan macromolecule, one HMGA links to two SDG units and, therefore, it is hypothesized that HMGA needs to be activated by CoA twice to make it possible to link two SDG molecules. Single CoA activated molecules are formed during cholesterol biosynthesis (Stryer, 1995), but no publications supporting the hypothesis of a di-activated HMGA are available. Therefore, linking of SDG to the second carboxyl group of HMGA, without prior activation by a second CoA, can not be excluded.
Furthermore it is hypothesized that, besides HMGA, other CoA-activated compounds with two carboxylic moieties, as malonic acid or glutaric acid, could act as linker molecules. The presence of other linker molecules could contribute to the identification of the so far unidentified peaks detected by MALDI-TOF MS (chapter 4). For flavonoids it is known that they can be malonylated (Suzuki et al., 2004). Since malonyl-CoA, just like HMGACoA, is formed as an intermediate of the fatty acid biosynthetic pathway (Stryer, 1995), the MALDI-TOF MS data were screened for molecules with malonyl moieties attached. No indications for other linker molecules than HMGA were found, since adducts of malonyl or other possible linker molecules and SDG could not be identified in the MALDI-TOF MS spectra.

## Advances in method development

## Extraction of lignans

Within this research, two extraction methods were used for the extraction of lignans from crude extracts, reaction mixtures or fermentation cultures. Dependent on the character of the molecules present, solid phase extraction (SPE; chapters 2, 3 and 4) or liquid-liquid partitioning (LQ-LQ; chapters 5 and 6) was used.
In Table 1, a comparison of the two extraction methods is shown. Samples (unstandardized) were extracted with both SPE and liquid-liquid partitioning with ethyl-acetate followed by analysis on analytical RP-HPLC. SDG is not extracted by liquid-liquid partitioning, while it is detected after SPE. On the other hand, the demethylated components (MHEND and DHEND) are best extracted by using liquid-liquid partitioning. For SECO, ENL and END hardly any differences between the two extraction techniques were observed. Between SECO and the demethylated compounds, differences in extraction efficiency by SPE were observed. Based on the chemical structures and their behavior on RP-HPLC, this was not expected. The $\log P$ values (Table 1) might help to explain these differences. It appears from Table 1 that compounds with a negative $\log P$ value will be best extracted by SPE. The difference in extraction efficiency between SECO and the demethylated compounds by SPE could not be explained based on the $\log P$ values.

Table 1. Comparison of peak area (AU) on RP-HPLC of lignans extracted by SPE and LQ-LQ extraction with ethyl acetate.

| Component | $\log P^{1}$ | LQ-LQ $^{2}$ | SPE $^{2}$ | Ratio LQ-LQ/SPE |
| :--- | :---: | :---: | :---: | :---: |
| CouAG | -0.8 | 722 | 1456 | 0.5 |
| FeAG | -0.8 | 435 | 1324 | 0.3 |
| SDG | -0.1 | 0.4 | 9.2 | $<0.1$ |
| DHEND | 2.4 | 2.9 | 0.3 | 9.8 |
| MHEND | 2.6 | 2.2 | trace | $>10$ |
| SECO | 2.9 | 5.3 | 5.2 | 1.0 |
| END | 2.9 | 0.8 | 0.6 | 1.3 |
| ENL | 3.3 | 0.3 | 0.2 | 1.6 |

${ }^{1} \log P$ was calculated by the Marvin software from Chemotaxon:
www.chemaxon.com/marvin/sketch/index.html
${ }^{2}$ The results were obtained by analyzing several different samples. These samples were not standardized.

## Analysis of lignans

The results of the MS analysis of lignans from sesame seeds by several ionization methods (chapter 6) lead to the conclusion that for the identification of all lignans in complex mixtures different ionization methods need to be combined.
With the current RP-HPLC-ESI-MS method, 77\% of the peak area of the fully saponified lignan macromolecule extract could be explained. The residual $23 \%$ of the peak area, representing 10-15 minor peaks, remained unidentified. Development of a multiple-ionization-method protocol for the identification of constituents of the lignan macromolecule might result in the identification of the so far unidentified peaks.

It is suggested that one of the unidentified peaks corresponds to FeA (as discussed above). Also other lignans as pinoresinol (PINO) and lariciresinol (LARI), which have been reported to occur in flaxseeds (Meagher et al., 1999; Milder et al., 2005a; Sicilia et al., 2003), are suggested to explain part of the unidentified peak area. Especially PINO might be detected by changing from negative to positive ionization mode, since in chapter 6 it was described that PINO was only detected by APCI-MS in the positive ionization mode and not in the negative ionization mode.
In addition, the analysis of fragments might be improved by making use of other separation techniques. Recently, the UPLC (ultra high pressure LC) was introduced as a technique that gives high-resolution separations and short analysis times. Another novel analytical separation technique is capillary electrophoresis (CE). The separation principle of CE is completely different from RP-HPLC and, therefore, might result in new identifications, especially when combined with MS.


Figure 2. Schematic representation of direct injection (A) and injection via at-column-dilution (B) for the preparative separation of lignans. The figure represents a situation at the start of a run. Example given: flow rate $=10 \mathrm{ml} / \mathrm{min}$, solvent composition at column entrance $=90 \%$ water $+10 \%$ acetonitrile.

## Preparative separation by at-column-dilution

Based on the results of chapter 5 it can be concluded that at-column-dilution (ACD) is a powerful tool for the purification of fermentation products. Initially direct injection was used (chapters 2 and 3) to apply a sample on a preparative RP-HPLC column. However, due to the low solubility of lignans in aqueous solutions, the loading volumes had to be extremely high (0.5-1.0 times the column volume), which resulted in peak broadening and
thus loss of resolution. Since the fermentation products were even less water-soluble than the plant lignans, injection via ACD was found to be a promising alternative (chapters 5 and 6). The principle of ACD is depicted in Fig. 2. Lignans are solubilized in an organic solvent in which the solubility of lignans is high resulting in a low loading volume. Just before the column, the organic phase containing the lignans is mixed with the aqueous phase (applied by a second pump) in such a ratio that the conditions applied on the column are the same as used for direct injection. Because of the mixing just before the column entrance, the lignans will not precipitate in the tubing and will be loaded on the column. Then, the gradient can be run as for direct injection. ACD allows 5-15 times lower loading volumes and because sample is injected in organic solvent, ACD makes it possible to apply relatively apolar components.

## Preparation of fermentation intermediates

## Conversion of lignans by single strain fermentation

Single strain bacteria have been used to prepare the demethylated and dehydroxylated intermediates of SECO and its dehydrated form, anhydrosecoisolariciresinol (AHS). As shown in chapter 5, mono- and di-demethylated intermediates could be obtained by incubations with P. productus, E. limosum or Cl. methoxybenzovorans. The dehydroxylated intermediates could be obtained by incubating the demethylated products with Eg. lenta.
It is concluded that AHS is demethylated in a similar way and to a similar extent as SECO. Furthermore, it is concluded that the dehydroxylation of AHS proceeds to a much lower extent than for SECO. The differences in structure of the aliphatic parts of the molecules are expected to be important factors in determining the substrate specificity of the dehydroxylases.

## Preparative preparation of (intermediate) fermentation products

A protocol has been developed by which all intermediate fermentation products of SECO and AHS can be obtained for their use in bioactivity studies. In this protocol the fermentation reaction was monitored by analytical RP-HPLC and stopped when similar amounts of the desired products were present. Then, the products were purified by semipreparative RP-HPLC loading the sample by at-column-dilution (experimental section chapter 5).
SECO and AHS were incubated by P. productus. The fermentation reaction was stopped after 12 h and 14 h , respectively. Mono- and di-demethylated products were purified by preparative RP-HPLC as indicated in Fig. 3. All compounds were baseline separated, resulting in the purification of each of them in a single run. The separation obtained resembles the separation obtained by analytical RP-HPLC (chapter 5), except for a shift in retention time caused by a difference in gradient. Analytical RP-HPLC analysis directly after fraction collection showed that the obtained demethylated compounds are between $95 \%$ and $97 \%$ pure based on peak area.


Figure 3. Preparative RP-HPLC profile of SECO (A) and AHS (B) demethylated by P. productus. The fermentation reaction was stopped after 12 and 14 h , respectively, to obtain both the mono- and di-demethylated intermediates.

In order to obtain dehydroxylated intermediates, purified di-demethylated SECO (DHEND) and di-demethylated AHS (DHENF) were subjected to fermentation by Eg. lenta. The reaction of DHEND was stopped after 76 h when equal amounts of DHEND and hydroxyenterodiol (HEND) were present (data not shown). The fermentation of DHENF with Eg. lenta showed only little conversion and the reaction was stopped after 93 h of incubation. The dehydroxylated compounds were also baseline separated by preparative RP-HPLC as shown in Fig. 4. The SECO-derived fermentation products were more than $92 \%$ pure based on analytical RP-HPLC analysis. The purity of hydroxy-enterofuran (HENF) and enterofuran (ENF) (the dehydroxylated products of DHENF) was less, most likely due to the low amounts present.


Figure 4. Preparative RP-HPLC profile of DHEND (A) and DHENF (B) dehydroxylated by Eg. lenta. The fermentation reaction of DHEND was stopped after 76 h (with 12 h of limited growth at 4 ${ }^{\circ} \mathrm{C}$ ) in order to obtain the mono-dehydroxylated intermediates. The fermentation of DHENF was stopped after 93 h of incubation.

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## Stability of lignans

Although the fermentation products obtained by preparative RP-HPLC separation with ACD were more than $92 \%$ pure, additional peaks were observed for the SECO-derived intermediates (accounting for $18-34 \%$ of the peak area) after concentration and storage. This indicates that the lignans were not stable under the conditions used. The concentration procedure consisted of the evaporation of acetonitrile and trifluoroacetic acid (TFA) at 70 ${ }^{\circ} \mathrm{C}$ and of a lyophilization step. The dry samples were stored in a desiccator. The factors, which might have contributed to the formation of the side products, are heat, acid and light. Under influence of light, photoisomerisation reactions can occur. This is shown in Fig. 5. CouAG, FeAG and SDG were purified from fully saponified lignan macromolecule and exposed to light for several weeks while they were dissolved in $20 \%$ ( $\mathrm{v} / \mathrm{v}$ ) acetonitrile acidified with $0.01 \%$ TFA. The samples were analyzed on RP-HPLC regularly. It was shown that the naturally present trans-CouAG and trans-FeAG (Struijs et al., 2007) were partially converted to their cis-isomers (as determined by NMR analysis; data not shown) until an equilibrium was reached (Fig. 5). Similarly, it was observed that from SDG a new peak was formed under influence of light. This peak was identified as a SDG dimer based on MS analysis ( $\mathrm{m} / \mathrm{z}=1369[\mathrm{M}-\mathrm{H}]^{+}$). These reactions did not occur when samples were stored in the dark. It remains unclear whether this isomerization reaction also can occur under water- limiting conditions.

Lignans are stable upon heating as shown in literature (Hyvarinen et al., 2006a; Strandas et al., 2008) and by the observation done during this study that the lignans in fermentation media were stable during sterilization ( $20 \mathrm{~min}, 140^{\circ} \mathrm{C}$; data not shown).
Heating under acidic conditions result in dehydration reactions as shown for the preparation of AHS (chapter 5). It is unclear what the acid concentration and the temperature should be for this reaction to occur. Dehydration was shown to take place at $70^{\circ} \mathrm{C}$ at a pH of 2.4 , because some of the side-products formed could be identified as dehydrated forms of the lignan originally present based on MS data.

## Estrogenicity of lignans

Lignans are structurally classified as phytoestrogens, because their chemical structure shows similarities with known estrogens like $17 \beta$-estradiol (Setchell et al., 1980; Waters and Knowler, 1982). In chapter 1 an overview is given about the estrogenic effects of various lignans (chapter 1, Table 3). The effects of the intermediate products formed during fermentation on the ER-mediated gene transcription pathway have not been investigated so far. Therefore, the interaction of the intermediates and end products of the fermentation of SECO and its dehydrated form, AHS with the human estrogen receptors ER $\alpha$ and ER $\beta$ was investigated.


Figure 5. Isomerisation of CouAG (A) and FeAG (B) and dimerization of SDG (C) under influence of light. Left panels: isomerisation and dimerisation in time. Right panels: RP-HPLC profiles of the incubations at the start of the incubation ( $\mathrm{t}=0$ days) and at the end ( $\mathrm{t}=73$ days).

## The estrogen receptor competitor assay

An estrogen receptor competitor assay (Invitrogen) was used to determine the relative binding to the ER $\alpha$ and ER $\beta$ of the intermediates and the mammalian lignans in comparison to $17 \beta$-estradiol (Sigma). In the assay, fluorescently labeled estradiol (fluoromone) is bound

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to the ER, forming a receptor-ligand cluster, which tumbles slowly in solution. The test compound is added and, dependent on its concentration and its binding affinity, displaces the fluoromone. Now, a slowly tumbling receptor-test compound cluster is present together with quickly tumbling fluoromone. Fluorescence polarization is determined as a measure for the tumbling rate of the fluorescent label (high polarization = slowly tumbling) and, therefore, indirectly is a measure for the amount of fluoromone displaced from the ER.

## Interaction of lignans with the estrogen receptor

The intermediates obtained by fermentation and commercial END and ENL were tested for their ability to displace fluoromone from the ER $\alpha$ and ER $\beta$. In order to be able to determine the $\mathrm{IC}_{50}$ value (concentration at which on $50 \%$ of the receptors, labeled estradiol is displaced by lignan), several concentrations were tested. The binding curves of the fermentation products were compared to those of estradiol. In Fig. 6, the curves of the displacement of estradiol, END and ENL with the ER $\alpha$ (Fig. 6A) and ER $\beta$ (Fig. 6B) are shown. In Table 2, the $\mathrm{IC}_{50}$ values are listed. When no interaction could be determined, the $\mathrm{IC}_{50}$ value is indicated as higher than ( $>$ ) the highest concentration tested.


Figure 6. Interaction of estradiol, END and ENL with ER $\alpha$ (A) and ER $\beta$ (B). Each curve is the average of four independent series. The error bars represent the standard deviation between four independent measurements.

Estradiol shows strong interaction with both the ER $\alpha$ and ER $\beta$. In accordance with literature, the interaction with ER $\alpha$ is slightly stronger than with ER $\beta$ (Mueller et al., 2004; Parker et al., 2000). ENL shows weak interaction with the ER $\beta\left(\mathrm{IC}_{50}=257 \mu \mathrm{M}\right.$ ), and hardly any interaction with the $\mathrm{ER} \alpha$ at the concentrations tested ( $\mathrm{IC}_{50}$ could not be determined). END shows no interaction with both estrogen receptors in the tested concentration range.
For the intermediate fermentation products, no displacement of labeled estradiol was observed for concentrations of $5 \mu \mathrm{M}$ or lower. For higher concentrations, the displacement could not be determined because of a background fluorescence signal interfering with the fluorescence polarization signal. The sample turned out to have a yellow-orange color, which is held responsible for the high background signal. This colored compound is suggested to originate either from the culture media or it is formed during sample work up.

Low levels of dimerized lignans could be formed under influence of light. The conjugated system is extended as a result of dimerization, which might result in color formation.

Table 2. IC $_{50}$ concentrations of END, ENL and estradiol for the displacement of estradiol on the ER $\alpha$ and ER $\beta$. The standard deviation represents the variation between four independent measurements.

| Component | ER $\alpha$ | ER $\beta$ |
| :--- | :---: | :---: |
| END | $>330 \mu \mathrm{M}$ | $>330 \mu \mathrm{M}$ |
| ENL | $>400 \mu \mathrm{M}$ | $257 \mu \mathrm{M} \pm 66$ |
| Estradiol | $9.4 \mathrm{nM} \pm 1.7$ | $22.6 \mathrm{nM} \pm 2.2$ |

## Structure-function relationships in ER-binding

The binding of a compound to the ER is determined by the fit of the molecule in the binding pocket, and thus by the chemical structure and conformation of the molecule. Important for binding of an estrogen to the ER is the presence of a phenolic hydroxyl at one end and a hydroxyl moiety at the other end of the molecule, and the volume of the molecule determined by its folding or conformation (Brzozowski et al., 1997).
END and ENL both contain the (phenolic) hydroxyl groups, which are important for binding. Therefore, it is suggested that conformational differences between these molecules determine their differences in ER-binding.
Crystal structures of lignans have shown that lignans can adopt a stacked or a stretched conformation. Crystal structures of SECO and AHS have been described (Fang et al., 1989; Milanesio et al., 1999), in which the two phenolic ring structures are stacked (Fig. 7). On the other hand, for a dibenzylic-8-hydroxylated lactone, a crystal structure has been described in which the molecule has a more linear conformation (Fig. 7) (Sefkow et al., 2001). For END and ENL no crystal structures have been described.

A


B


C


Figure 7. 3D structures of secoisolariciresinol (A), anhydrosecoisolariciresinol (B) and dibenzylic-8hydroxylated lactone (C) as reported in literature (Fang et al., 1989; Milanesio et al., 1999; Sefkow et al., 2001).

It is hypothesized that the stacked structure is too bulky and, therefore, does not fit in the narrow cleft of the ligand binding domain (LBD) of the ER, whereas the linear

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conformation does. Since the structure of the aliphatic part of ENL resembles the structure of the aliphatic part of the dibenzylic-8-hydroxylated lactone (Fig. 7C), it is suggested that ENL adopts a more linear conformation. The structure of the aliphatic part of END, is similar to those of SECO and, therefore, END is expected to adopt a more stacked conformation. The fact that for ENL interaction with the ER $\beta$ was observed while END did not bind, supports the hypothesis that stacked lignans do not bind and linear lignans bind to the LBD. Further experiments should confirm the hypothesis that ENL has a linear, and END has a stacked conformation.
A related question is how the decoration of the aromatic rings influences the conformation. Upon fermentation, methyl and hydroxyl groups are removed from the plant lignans. These groups are involved in hydrogen bonding, which have been suggested to play a role in the formation or stabilization of the stacked conformation of SECO (Milanesio et al., 1999). Removal of the methyl and hydroxyl groups is expected to result in a destabilization of the stacked conformation. It is unclear whether this destabilization will assist in the stretching of the molecules or that stacking or stretching is completely determined by the aliphatic part of the molecule.

## Estrogenicity of lignans via other mechanisms than ER-binding

The results presented above and the results of other studies (Mueller et al., 2004; Penttinen et al., 2007) show that the estrogenic potential of lignans by ER-binding is low. By measuring only ER binding, subsequent steps in the ER-mediated gene transcription cascade, as binding to the ERE and coactivator recruitment, are neglected. Furthermore, no discrimination between agonists and antagonists can be made. Transfected cell line assay in which human cells or yeasts are transfected with ER and with a reporter gene construct, measure the complete cascade.
Besides this ER-mediated gene transcription cascade, estrogenic effects can also be induced by influencing the estradiol biosynthetic pathway and by influencing the estrogen transport via SHBG (Adlercreutz et al., 1987; Hillerns et al., 2005; McCann et al., 2007; Schöttner et al., 1997). Therefore, the influence of lignans on SHBG activation or binding, and on the estradiol biosynthesis should be investigated to determine, which mechanism is most important for the estrogenic effects of lignans. Furthermore, the relevance of each of the mechanisms at physiologic concentrations should be investigated.

## The importance of the enterohepatic circulation

In vivo studies show that sesamin can be converted into the mammalian lignans END and ENL (Coulman et al., 2005; Liu et al., 2006; Penalvo et al., 2005). However, in analogy with another in vitro study, in chapter 7, only small amounts of mammalian lignans were formed upon in vitro fermentation of a sesame lignan extract (Liu et al., 2006). In addition to the demethylation and dehydroxylation steps, as shown for the conversion of SECO and MAT, the furan moieties and the methylenedioxybridges need to be cleaved during the conversion of sesame lignans to mammalian lignans. The furan ring structures can be cleaved by bacteria from the human intestinal flora (Heinonen et al., 2001). For the
cleavage of the methylenedioxybridges, it has been suggested that liver metabolism during enterohepatic circulation is essential (Nakai et al., 2003). The results of the fermentation study described in chapter 6, in which PINO was converted to mammalian lignans while sesamin or sesaminol were not fermented, support the role of liver metabolism and enterohepatic circulation in the conversion of methylenedioxybridged furanofuran lignans into mammalian lignans.

The enterohepatic circulation is also found to be important in the metabolism of the dibenzylbutane lignans. Besides the plant lignans and the mammalian lignans, also a range of hydroxylated metabolites of END and ENL have been identified in human plasma or urine (Smeds et al., 2006). The hydroxylated metabolites were also identified after administration of liver microsomes with lignans (Niemeyer et al., 2003; Smeds et al., 2005), and are, therefore, suggested to be the result of phase I detoxification reactions of the liver. Enterohepatic circulation also explains the relatively stable plasma levels of mammalian lignans (Nesbitt et al., 1999).
A schematic overview of mammalian lignans in the enterohepatic circulation is given in Fig. 8. The ingested lignans reach the colon where they are subjected to fermentation (Fig. 8A). The fermentation products (and some unfermented plant lignans) are absorbed. Already during absorption, they undergo phase II detoxification reaction resulting in glucuronide or sulfate conjugates (Jansen et al., 2005). More than $95 \%$ of all lignans in human body fluids are present in a conjugated form of which about $80 \%$ is glucuronidated (Axelson and Setchell, 1980; Axelson and Setchell, 1981; Jansen et al., 2005). The conjugated lignans are distributed throughout the human body. In the liver, as a result of phase I detoxification, they are hydroxylated (Dean et al., 2004; Jacobs et al., 1999; Niemeyer et al., 2003). Hydroxylation has been observed both at the phenolic ring and at the aliphatic part of the molecule (Jacobs et al., 1999; Niemeyer et al., 2003). The metabolites are excreted via the bile into the colon where they start their succeeding cycle of enterohepatic circulation (Fig. 8B). In the colon, part of the metabolites are deconjugated by the intestinal microflora liberating mammalian lignans, plant lignans and hydroxylated mammalian lignans. Another part of the metabolites, which enter the colon via the bile, is excreted as such by the feces. Deconjugation makes the lignans again available for absorption or for further microbial conversion. It should be noted that lignans also might be degraded completely due to microbial action and that part of the conjugated lignans are excreted via the urine.
The hydroxylated metabolites might be responsible for the increased estrogenicity as observed by Pentinnen et al. (2007) as a result of cellular metabolism. Besides, the molecules are hydroxylated at other positions than the fermentation intermediates and therefore, knowledge on their estrogenicity will contribute to the understanding of the structure-function relationships of the estrogenicity of lignans. It should be noted that upon absorption of lignans by target tissue cells, the conjugated lignans are thought to be deconjugated, which makes the deconjugated compounds responsible for the bioactive responses.

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Figure 8. Schematic overview of SECO in the enterohepatic circulation. (A) The first cycle of the enterohepatic circulation. SECO enters the colon and is fermented into END and ENL, dependent on the composition of the microflora. During absorption lignans are glucuronidated or sulfated (END ${ }^{\mathrm{G}}=$ glucuronide of END; sulfated conjugates are less present and are therefore not depicted) and via the blood lignans are transported to body tissues or to the liver. In the liver lignans are hydroxylated (HOEND; OH-ENL) as a result of phase I metabolism and secreted via the bile. (B) Succeeding cycles of the enterohepatic circulation. Via the bile, metabolized lignans enter the colon, they can be fermented and re-absorbed or leave the body via the feces. Lignans are also excreted via the urine (not shown) and they can be fermented to non-phenolic component. SECO $^{G}$ ? $=$ it is unknown if $\mathrm{SECO}^{\mathrm{G}}$ is metabolized in the liver. It is unknown whether intermediate fermentation products undergo enterohepatic circulation as well.

## Lignans as functional food ingredient

The average lignan consumption in The Netherlands is about 1 mg per day ( $\sim 3 \mu \mathrm{~mol}$ when consumed in the form of SECO) (Milder et al., 2005b), which results in average plasma concentrations of $0.09 \mathrm{nmol} / \mathrm{I}$ END and $0.18 \mathrm{nmol} / \mathrm{I}$ ENL (Kuijsten, 2007). Independent of the mechanism of estrogenicity, for estrogenic responses of lignans concentrations in the micromolar range are needed (chapter 1, Table 3). Therefore, consumption of lignans via the normal diet seems to be insufficient. To increase consumption of lignans, foods, as bread or dairy products, can be fortified (Hyvarinen et al., 2006a; Hyvarinen et al., 2006b; Strandas et al., 2008). This will lead to an increase in intake, but it is expected that this increase is not large enough to result in bioactive responses. Lignans can also be consumed as supplements. Both plant lignans and mammalian lignans can be used as a lignan source.

## Lignan macromolecule as supplement

Supplements containing extracts of flaxseed lignans, mainly in the form of the lignan macromolecule, are currently on the market. Lignan macromolecule needs to be digested to monomeric lignans in the human body. Subsequently, they need to be fermented before bioactive compounds are released.
The lignan macromolecule reaches the colon intact (Eeckhaut et al., 2008) assuming that the bacterial flora and the brush border enzymes of the small intestine do not degrade it. In the colon, the lignan macromolecule is degraded and eventually SECO, CouA, FeA, herbacetin and HMGA are released. Subsequently, fermentation by the bacteria of the human intestinal flora takes place. There are, however, large interindividual differences in microbial composition, which result in large interindividual variations in the mammalian lignan production (Kilkkinen et al., 2001; Possemiers et al., 2007). So even though lignan macromolecule is targeted to the colon, the bioacive potential of the lignan macromolecule may vary from person to person. This makes it difficult to predict the implications of lignan macromolecule on human health.

This prediction is further complicated by the presence of other bioactive constituents than lignans in the lignan macromolecule. FeA and CouA have been shown to have antioxidant activity at similar concentrations as lignans (Ferguson et al., 2005; Fukumoto and Mazza, 2000; Garcia-Conesa et al., 1999). Even though flavonoids are minor constituents, they also may contribute to the antioxidant capacity of the lignan macromolecule (Fukumoto and Mazza, 2000; Rice-Evans et al., 1996). HMGA, being a precursor of cholesterol and an intermediate in ketogenesis (Stryer, 1995), plays a key role in lipid metabolism. A plasma concentration of a healthy subject is about $46 \mu \mathrm{~mol} / \mathrm{l}$ (Lippe et al., 1987). No studies on the effect of dietary HMGA have been reported. Assuming an intake of about $0.5 \mu \mathrm{~mol}$ per day (all lignan intake is in the form of lignan macromolecule and $10 \%$ thereof is HMGA), no large effects are expected.
All constituents contributing to the health effects makes that lignan macromolecule is a complex extract of which the precise effects are hard to predict.

## SECO and SDG as supplement

Since lignan macromolecule is a less strictly defined source, purified SECO or SDG could be used as an alternative lignan source. However, also SECO and SDG should be bioactivated in the human intestinal tract. Their biologic responses are, just as the response of lignan macromolecule, dependent on the interindividual variations in the composition of the human intestinal flora. In addition, the enterohepatic circulation seems to play an important role in their bioactivity.
For SDG it has been shown that it is stable under conditions in stomach and small intestine and it has been suggested to reach the colon intact (Clavel et al., 2006a). SDG will not be absorbed before it is deglucosylated, as shown for (iso)flavonoids (Nemeth et al., 2003; Setchell et al., 2002). Therefore, it is expected that SDG is targeted to the colon, although it also might be deglucosylated by enzymes of the small intestine (Nemeth et al., 2003) and absorbed (partially) before it reaches the colon.
Even though SDG was found to be stable under acidic conditions (Clavel et al., 2006a), the stability of SECO in the stomach is not clear. SECO is susceptible for dehydration under acidic conditions, especially at elevated temperature, but it is unclear if this reaction takes place in the stomach. Besides, SECO is not targeted to the colon. Since SECO can be detected in human plasma as such, it is expected to be (partially) absorbed early in the gastro-intestinal tract (Penalvo et al., 2005).
When absorbed before the colon is reached, SECO is expected to enter the enterohepatic circulation. After excretion via the bile, it will end up in the colon where it can be fermented into the mammalian lignans after all. It is expected that part of SECO will be excreted (via urine and feces) before it is fermented, making SECO an inefficient source of lignans. This also holds for SDG. Nevertheless, a part of the SDG ingested is expected to reach the colon, which improves its efficiency compared to SECO. Besides, for both SECO and SDG the interindividual variations in microflora have a large impact on their estrogenic effects, and are, therefore, also not considered as the most optimal lignans source for supplementation.

## Mammalian lignans as supplement

By making use of mammalian lignans as supplement, the interindividual variations in the microflora do not influence the bioavailability of the lignans.
An ex vivo procedure for the production of mammalian lignans is needed to be able to use mammalian lignans in supplements. The mammalian lignan END can be formed by the combined action of a demethylating and a dehydroxylating bacteria. P. productus, E. limosum and Cl . methoxybenzovorans were identified as demethylating bacteria (chapter 5). However, E. limosum, being a risk group 2 bacteria, is not a bacteria that can be used for the production of mammalian lignans for consumption. Eg. lenta was the only strain tested that could dehydroxylate lignans. Even though this bacterium was identified in human feces (Jin et al., 2007b; Wang et al., 2000), it is classified as a risk group 2 bacterium. So for the commercial production of END, another bacterium should be identified, which can dehydroxylate lignans. Although Cl. scindens did not dehydroxylate demethylated SECO in this study, it has been shown to be able to convert SECO into END in a co-incubation with P. productus (Clavel et al., 2005), which makes Cl. scindens a suitable candidate for the dehydroxylation reaction.

As a final step of the fermentation reaction, END should be dehydrogenated into ENL. This fermentation step was not investigated in this study. Several bacteria able to convert END into ENL have been described in literature (Clavel et al., 2006a; Clavel et al., 2007; Jin et al., 2007a).
Even though the conversion of SECO to ENL consists of three steps, each step requiring a specific bacterium, it might be possible to co-incubate SECO with three bacterial strains. The growth conditions for the bacteria used in this and other studies (Clavel et al., 2006b; Jin et al., 2007a) were similar although the growth rates differed. It should, therefore, be investigated if all bacteria can grow when co-incubated.
No direct information about the absorption of mammalian lignans is available. Based on the identification of plant lignans in plasma and urine (Penalvo et al., 2005), it is expected that also the mammalian lignans can be absorbed as such. They might even already be absorbed in the small intestine. The bioactivity of mammalian lignans is, anyhow, not dependent on targeting to the colon. The stability of mammalian lignans under the acidic conditions in the stomach should be investigated.

Thus, mammalian lignans seem to be a better lignan source for the use in supplements compared to lignan macromolecule, SDG and SECO. However, the digestion and absorption of mammalian lignans should be studied further and safer dehydroxylating bacteria should be identified. Furthermore, a procedure should be developed for the large scale extraction of the mammalian lignans from the fermentation culture. At laboratory scale, liquid-liquid partitioning followed by RP-HPLC separation was used to extract the mammalian lignans.

In conclusion, this thesis research resulted in a more detailed characterization of the lignan macromolecule from flaxseed hulls and its bioconversion. The impact of the various constituents of the lignan macromolecule and their conversion products on human health was discussed.

## References

Adlercreutz, H., Höckerstedt, K., Bannwart, C., Bloigu, S., Hämäläinen, E., Fotsis, T., Ollus, A., 1987. Effect of dietary components, including lignans and phytoestrogens, on enterohepatic circulation and liver metabolism of estrogens and on sex hormone binding globulin (SHBG). J. Steroid Biochem. 27, 1135-1144.

Axelson, M., Setchell, K.D.R., 1980. Conjugation of Lignans in Human-Urine. FEBS Lett. 122, 49-53.

Axelson, M., Setchell, K.D.R., 1981. The excretion of lignans in rats - evidence for an intestinal bacterial source for this new group of compounds. FEBS Lett. 123, 337-342.

Brzozowski, A.M., Pike, A.C.W., Dauter, Z., Hubbard, R.E., Bonn, T., Engström, O., Öhman, L., Greene, G.L., Gustafsson, J.Å., Carlquist, M., 1997. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389, 753-758.

Clavel, T., Henderson, G., Alpert, C.A., Philippe, C., Rigottier-Gois, L., Dore, J., Blaut, M., 2005. Intestinal bacterial communities that produce active estrogen-like compounds enterodiol and enterolactone in humans. Appl. Environ. Microbiol. 71, 6077-6085.

Clavel, T., Borrmann, D., Braune, A., Dore, J., Blaut, M., 2006a. Occurrence and activity of human intestinal bacteria involved in the conversion of dietary lignans. Anaerobe 12, 140147.

Clavel, T., Henderson, G., Engst, W., Dore, J., Blaut, M., 2006b. Phylogeny of human intestinal bacteria that activate the dietary lignan secoisolariciresinol diglucoside. FEMS Microbiol. Ecol. 55, 471-478.

Clavel, T., Lippman, R., Gavini, F., Dore, J., Blaut, M., 2007. Clostridium saccharogumia sp nov and Lactonifactor longoviformis gen. nov., sp nov., two novel human faecal bacteria involved in the conversion of the dietary phytoestrogen secoisolariciresinol diglucoside. Syst. Appl. Microbiol. 30, 16-26.

Coulman, K.D., Liu, Z., Hum, W.Q., Michaelides, J., Thompson, L.U., 2005. Whole sesame seed is as rich a source of mammalian lignan precursors as whole flaxseed. Nutr. Canc. Int. J. 52, 156-165.

Dean, B., Chang, S., Doss, G.A., King, C., Thomas, P.E., 2004. Glucuronidation, oxidative metabolism, and bioactivation of enterolactone in rhesus monkeys. Arch. Biochem. Biophys. 429, 244-251.

Eeckhaut, E., Struijs, K., Possemiers, S., Vincken, J.-P., De Keukeleire, D., Verstraete, W., 2008. Metabolism of the lignan macromolecule into enterolignans in the gastrointestinal
lumen as determined in the simulator of the human intestinal microbial ecosystem. J. Agric. Food Chem. 56, 4806-4812.

Fang, J.M., Hsu, K.C., Cheng, Y.S., 1989. Lignans from leaves of Calocedrus-Formosana. Phytochemistry 28, 3553-3555.

Ferguson, L.R., Zhu, S.T., Harris, P.J., 2005. Antioxidant and antigenotoxic effects of plant cell wall hydroxycinnamic acids in cultured HT-29 cells. Mol. Nutr. Food Res. 49, 585-593.

Ford, J.D., Huang, K.S., Wang, H.B., Davin, L.B., Lewis, N.G., 2001. Biosynthetic pathway to the cancer chemopreventive secoisolariciresinol diglucoside-hydroxymethyl glutaryl ester- linked lignan oligomers in flax (Linum usitatissimum) seed. J. Nat. Prod. 64, 1388-1397.

Fukumoto, L.R., Mazza, G., 2000. Assessing antioxidant and prooxidant activities of phenolic compounds. J. Agric. Food Chem. 48, 3597-3604.

Garcia-Conesa, M.T., Wilson, P.D., Plumb, G.W., Ralph, J., Williamson, G., 1999. Antioxidant properties of 4,4 '-dihydroxy-3,3 '-dimethoxy-beta,beta '-bicinnamic acid (8-8diferulic acid, non-cyclic form). J. Sci. Food Agric. 79, 379-384.

Heinonen, S., Nurmi, T., Liukkonen, K., Poutanen, K., Wahala, K., Deyama, T., Nishibe, S., Adlercreutz, H., 2001. In vitro metabolism of plant lignans: New precursors of mammalian lignans enterolactone and enterodiol. J. Agric. Food Chem. 49, 3178-3186.

Hillerns, P.I., Zu, Y.G., Fu, Y.J., Wink, M., 2005. Binding of phytoestrogens to rat uterine estrogen receptors and human sex hormone-binding globulins. Z. Naturforsch.(C) 60, 649656.

Hyvarinen, H.K., Pihlava, J.M., Hiidenhovi, J.A., Hietaniemi, V., Korhonen, H.J.T., Ryhanen, E.L., 2006a. Effect of processing and storage on the stability of flaxseed lignan added to bakery products. J. Agric. Food Chem. 54, 48-53.

Hyvarinen, H.K., Pihlava, J.M., Hiidenhovi, J.A., Hietaniemi, V., Korhonen, H.J.T., Ryhanen, E.L., 2006b. Effect of processing and storage on the stability of flaxseed lignan added to dairy products. J. Agric. Food Chem. 54, 8788-8792.

Jacobs, E., Kulling, S.E., Metzler, M., 1999. Novel metabolites of the mammalian lignans enterolactone and enterodiol in human urine. J. Steroid Biochem. Mol. Biol. 68, 211-218.

Jansen, G.H.E., Arts, I.C.W., Nielen, M.W.F., Muller, M., Hollman, P.C.H., Keijer, J., 2005. Uptake and metabolism of enterolactone and enterodiol by human colon epithelial cells. Arch. Biochem. Biophys. 435, 74-82.

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Jin, J.S., Kakiuchi, J., Hattori, M., 2007a. Enantioselective oxidation of enterodiol to enterolactone by human intestinal bacteria. Biol. Pharm. Bull. 30, 2204-2206.

Jin, J.S., Zhao, Y.F., Nakamura, N., Akao, T., Kakiuchi, N., Min, B.S., Hattori, M., 2007b. Enantioselective dehydroxylation of enterodiol and enterolactone precursors by human intestinal bacteria. Biol. Pharm. Bull. 30, 2113-2119.

Kilkkinen, A., Stumpf, K., Pietinen, P., Valsta, L.M., Tapanainen, H., Adlercreutz, H., 2001. Determinants of serum enterolactone concentration. Am. J. Clin. Nutr. 73, 10941100.

Li, X., Yuan, J.P., Xu, S.P., Wang, J.H., Liu, X., 2008. Separation and determination of secoisolariciresinol diglucoside oligomers and their hydrolysates in the flaxseed extract by high-performance liquid chromatography. J. Chromatogr. A 1185, 223-232.

Lippe, G., Trevisan, R., Nosadini, R., Fabris, R., Deana, R., 1987. 3-Hydroxy-3Methylglutaric, Adipic, and 2-Oxoglutaric Acids Measured by Hplc in the Plasma from Diabetic-Patients. Clin. Biochem. 20, 275-279.

Liu, Z., Saarinen, N.M., Thompson, L.U., 2006. Sesamin is one of the major precursors of mammalian lignans in sesame seed (Sesamum indicum) as observed in vitro and in rats. J. Nutr. 136, 906-912.

Madhusudhan, B., Wiesenborn, D., Schwarz, J., Tostenson, K., Gillespie, J., 2000. A dry mechanical method for concentrating the lignan secoisolariciresinol diglucoside in flaxseed. Food. Sci. Technol. 33, 268-275.

McCann, S.E., Wactawski-Wende, J., Kufel, K., Olson, J., Ovando, B., Kadlubar, S.N., Davis, W., Carter, L., Muti, P., Shields, P.G., Freudenheim, J.L., 2007. Changes in 2hydroxyestrone and 16 alpha-hydroxyestrone metabolism with flaxseed consumption: Modification by COMT and CYP1B1 genotype. Cancer Epidemiol. Biomarkers Prev. 16, 256-262.

Meagher, L.P., Beecher, G.R., Flanagan, V.P., Li, B.W., 1999. Isolation and characterization of the lignans, isolariciresinol and pinoresinol, in flaxseed meal. J. Agric. Food Chem. 47, 3173-3180.

Milanesio, M., Viterbo, D., Chattopadhyay, S.K., Kulshrestha, M., Appendino, G., 1999. Crystal and molecular structure of secoisolariciresinol. Croat. Chem. Acta 72, 351-363.

Milder, I.E.J., Arts, I.C.W., van de Putte, B., Venema, D.P., Hollman, P.C.H., 2005a. Lignan contents of Dutch plant foods: a database including lariciresinol, pinoresinol, secoisolariciresinol and matairesinol. Br. J. Nutr. 93, 393-402.

Milder, I.E.J., Feskens, E.J.M., Arts, I.C.W., de Mesquita, H.B.B., Hollman, P.C.H., Kromhout, D., 2005b. Intake of the plant lignans secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol in Dutch men and women. J. Nutr. 135, 1202-1207.

Mueller, S.O., Simon, S., Chae, K., Metzler, M., Korach, K.S., 2004. Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor alpha (ER alpha) and ER beta in human cells (vol 80, pg 14, 2004). Toxicol. Sci. 81, 530-531.

Nakai, M., Harada, M., Nakahara, K., Akimoto, K., Shibata, H., Miki, W., Kiso, Y., 2003. Novel antioxidative metabolites in rat liver with ingested sesamin. J. Agric. Food Chem. 51, 1666-1670.

Nemeth, K., Plumb, G.W., Berrin, J.G., Juge, N., Jacob, R., Naim, H.Y., Williamson, G., Swallow, D.M., Kroon, P.A., 2003. Deglycosylation by small intestinal epithelial cell betaglucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans. Eur. J. Nutr. 42, 29-42.

Nesbitt, P.D., Lam, Y., Thompson, L.U., 1999. Human metabolism of mammalian lignan precursors in raw and processed flaxseed. Am. J. Clin. Nutr. 69, 549-555.

Niemeyer, H.B., Honig, D.M., Kulling, S.E., Metzler, M., 2003. Studies on the metabolism of the plant lignans secoisolariciresinol and matairesinol. J. Agric. Food. Chem. 51, 63176325.

Parker, G.J., Law, T.L., Lenoch, F.J., Bolger, R.E., 2000. Development of high throughput screening assays using fluorescence polarization: Nuclear receptor-ligand-binding and kinase/phosphatase assays. J. Biomol. Screen 5, 77-88.

Penalvo, J.L., Heinonen, S.M., Aura, A.M., Adlercreutz, H., 2005. Dietary sesamin is converted to enterolactone in humans. J. Nutr. 135, 1056-1062.

Penttinen, P., Jaehrling, J., Damdimopoulos, A.E., Inzunza, J., Lemmen, J.G., van der Saag, P., Pettersson, K., Gauglitz, G., Makela, S., Pongratz, I., 2007. Diet-derived polyphenol metabolite enterolactone is a tissue-specific estrogen receptor activator. Endocrinology 148, 4875-4886.

Possemiers, S., Bolca, S., Eeckhaut, E., Depypere, H., Verstraete, W., 2007. Metabolism of isoflavones, lignans and prenylflavonoids by intestinal bacteria: producer phenotyping and relation with intestinal community. FEMS Microbiol. Ecol. 61, 372-383.

Rice-Evans, C.A., Miller, N.J., Paganga, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids (vol 20, pg 933, 1996). Free Radic. Biol. Med. 21, 417-417.

Schöttner, M., Gansser, D., Spiteller, G., 1997. Lignans from the roots of Urtica dioica and their metabolites bind to human sex hormone binding globulin (SHBG). Planta Med. 63, 529-532.

Sefkow, M., Kelling, A., Schilde, U., 2001. Enantioselective synthesis of alphahydroxylated enterolactone and analogs. Tetrahedron Lett. 42, 5101-5104.

Setchell, K.D.R., Lawson, A.M., Mitchell, F.L., Adlercreutz, H., Kirk, D.N., Axelson, M., 1980. Lignans in man and in animal species. Nature 287, 740-742.

Setchell, K.D.R., Brown, N.M., Zimmer-Nechemias, L., Brashear, W.T., Wolfe, B.E., Kirschner, A.S., Heubi, J.E., 2002. Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. Am. J. Clin. Nutr. 76, 447-453.

Sicilia, T., Niemeyer, H.B., Honig, D.M., Metzler, M., 2003. Identification and stereochemical characterization of lignans in flaxseed and pumpkin seeds. J. Agric. Food Chem. 51, 1181-1188.

Smeds, A.I., Saarinen, N.M., Eklund, P.C., Sjholm, R.E., Makela, S.I., 2005. New lignan metabolites in rat urine. J. Chromatogr. B 816, 87-97.

Smeds, A.I., Hakala, K., Hurmerinta, T.T., Kortela, L., Saarinen, N.M., Makela, S.I., 2006. Determination of plant and enterolignans in human serum by high-performance liquid chromatography with tandem mass spectrometric detection. J. Pharm. Biomed. Anal. 41, 898-905.

Smeds, A.I., Eklund, P.C., Sjoholm, R.E., Willfor, S.M., Nishibe, S., Deyama, T., Holmbom, B.R., 2007. Quantification of a broad spectrum of lignans in cereals, oilseeds, and nuts. J. Agric. Food Chem. 55, 1337-1346.

Strandas, C., Kamal-Eldin, A., Andersson, R., Aman, P., 2008. Phenolic glucosides in bread containing flaxseed. Food Chem. 110, 997-999.

Struijs, K., Vincken, J.-P., Verhoef, R., van Oostveen-van Casteren, W.H.M., Voragen, A.G.J., Gruppen, H., 2007. The flavonoid herbacetin diglucoside as a constituent of the lignan macromolecule from flaxseed hulls. Phytochemistry 68, 1227-1235.

Suzuki, H., Nakayama, T., Nagae, S., Yamaguchi, M.A., Iwashita, T., Fukui, Y., Nishino, T., 2004. cDNA cloning and functional characterization of flavonol 3-O-glucoside-6 "-Omalonyltransferases from flowers of Verbena hybrida and Lamium purpureum. J. Mol. Catal. B-Enzym. 28, 87-93.

Wang, L.-Q., Meselhy, M.R., Li, Y., Qin, G.-W., Hattori, M., 2000. Human intestinal bacteria capable of transforming secoisolariciresinol diglucoside to mammalian lignans, enterodiol and enterolactone. Chem. Pharm. Bull. 48, 1606-1610.

Waters, A.P., Knowler, J.T., 1982. Effect of a lignan (HPMF) on RNA synthesis in the rat uterus. J. Reprod. Fertility 66, 379-381.

2.

## Summary

Lignans are diphenolic compounds, which are functionally classified as phytoestrogens, because of their structural similarities to estradiol and other known estrogenic compounds. Because of their interaction with the human estrogen metabolism, and because of their antioxidant activities, lignans have been related to several health beneficial effects.
Plant lignans can be converted into the so-called mammalian lignans enterodiol (END) and enterolactone (ENL) by the bacteria in the human colon. These mammalian lignans are held responsible for the health effects, since they are the predominant circulating lignans in the human body.
The lignans in flaxseeds are linked within the so-called lignan macromolecule. Despite the fact that the composition of the lignan macromolecule from flaxseed has been investigated, its structure is largely unknown. Furthermore, it is known that lignans are converted into the bioactive mammalian lignans in the colon, but protocols to produce these lignans by single strain fermentations are sparsely available as are efficient protocols for their recovery from fermentation cultures. In order to address these points, the aims of the research described in this thesis are to identify the precise composition and structure of the lignan macromolecule from flaxseeds, to convert the plant lignans into the mammalian lignans by fermentation, and to investigate how the bioconversion of lignans influences their estrogenicity. In order to be able to reach these goals, analytical and preparative protocols need to be developed.

In chapter 1, a general introduction is given on the biosynthesis, the bioconversion and the bioactivity of lignans. The lignans from flaxseeds and sesame seeds are described, together with a literature review on how these lignans influence human health with special emphasis on estrogenicity and antioxidant activity. Furthermore, the (tentative) fermentation pathways of lignans from flaxseeds and sesame seeds into mammalian lignans are described.
In chapters 2-4, the structure of the lignan macromolecule from flaxseed hulls was investigated. Flaxseed hulls were used because their lignan content was even higher compared to whole seeds. In chapter 2, the lignan macromolecule was degraded to its monomeric units by saponification with 75 mM NaOH . Besides the lignan secoisolariciresinol diglucoside (SDG), the hydroxycinnamic acid glucosides, p-coumaric acid glucoside (CouAG) and ferulic acid glucoside (FeAG), the flavonoid herbacetin diglucoside (HDG) was identified as a novel constituent of the lignan macromolecule as determined by RP-HPLC-MS, and confirmed by NMR after preparative separation of the monomeric constituents.
Further proof for HDG as a constituent of the lignan macromolecule, was obtained by fragmentation of the lignan macromolecule by partial saponification, and subsequent separation by RP-HPLC. A fragment consisting of HDG and 3-hydroxy-3-methyl glutaric acid (HMGA) could be identified based on MS analysis. This fragment was isolated by preparative RP-HPLC and its identity was confirmed by NMR. This showed that HDG is incorporated in the lignan macromolecule via the same linker molecule as SDG.
Partial saponification appeared to be an essential tool to obtain fragments of the lignan macromolecule for its structural characterization, since more key fragments of the lignan
macromolecule were obtained following this treatment prior to preparative RP-HPLC separation. The fragments obtained were used to identify how CouAG and FeAG are linked within the lignan macromolecule. As described in chapter 3, CouAG and FeAG were shown to be linked directly to the lignan macromolecule via their carboxylic moieties. A fragment in which CouAG was linked to the C-6 position of one of the glucosyl moieties of SDG was identified, but a similar fragment for FeAG could not be isolated. Besides, a fragment, in which ferulic acid (FeA) was linked to SDG, was identified. In contrast to CouAG, FeA linked to SDG via the C-2 position of one of the glucosyl moieties.
In chapter 4, the composition and size of the lignan macromolecule was investigated. The linker molecule HMGA was quantified based on GC data, and found to contribute for $11 \%$ ( $\mathrm{w} / \mathrm{w}$ ) to the lignan macromolecule. The molar extinction coefficients of SDG, HDG, CouAG and FeAG at 280 nm , were determined to be 5838, 10347, 19474, and $12133 \mathrm{M}^{-}$ ${ }^{1} \mathrm{~cm}^{-1}$, respectively. Based on these values and RP-HPLC data, it was determined that SDG contributes for $56 \mathrm{~mol} \%$ to the phenolic constituents of the lignan macromolecule, while CouAG, FeAG, and HDG contribute for 23, 15 and $6 \mathrm{~mol} \%$, respectively.
In order to obtain data on the (average) molecular mass of the lignan macromolecule, it was analyzed and fractionated by gel permeation chromatography. Due to a lack of standards, no direct data on the molecular mass could be obtained. Nevertheless, the broad peak indicated that the lignan macromolecule should be defined as a collection of molecules differing in molecular mass. The subunit composition of the fractions collected was determined by RP-HPLC after full saponification. It was shown that the proportion hydroxycinnamic acid glucosides (= CouAG + FeAG) is negatively correlated with the molecular mass. This led to the hypothesis that incorporation of CouAG and FeAG into the lignan macromolecule resulted in chain termination. This might suggest that the amount of hydroxycinnamic acid glucosides present during biosynthesis determines the chain length of the lignan macromolecule.
Analysis of the lignan macromolecule by MALDI-TOF MS showed a complex pattern of peaks, which showed that molecules consisting of at least 5 SDG and 5 HMGA moieties are part of the lignan macromolecule.
A model describing the chain length of the lignan macromolecule was developed based on the structural and compositional data. The average chain length was calculated to be 3 SDG moieties with 2 hydroxycinnamic acid glucosides at the terminal positions, with a variation between 1 and 7 SDG moieties.

In chapter 5, secoisolariciresinol (SECO) was obtained by enzymatic deglucosylation and anhydrosecoisolariciresinol (AHS) by acid treatment of fully saponified lignan macromolecule. SECO and AHS differ in the aliphatic part of their structure. SECO carries a diol structure, while in AHS this diol structure is dehydrated into a furan ring. They were both subjected to fermentation by single strain bacteria, which have been identified as members of the human intestinal flora or closely related species. Both SECO and AHS were demethylated by Peptostreptococcus productus, Eubacterium limosum and Clostridium methoxybenzovorans. First mono-demethylated products were formed followed by the didemethylated compounds.
Di-demethylated SECO and AHS were obtained by a large batch fermentation of SECO and AHS followed by preparative RP-HPLC purification. Di-demethylated SECO was
dehydroxylated by Eggerthella lenta, which resulted in the formation of END. Upon incubation of di-demethylated AHS with Eggerthella lenta small quantities of dehydroxylated reaction products were formed, but the rate at which di-demethylated AHS was dehydroxylated was much lower than that of di-demethylated SECO.
These conversion reactions by bacteria form the human intestinal flora resulted in two series of lignans, which differ in the structure of the aliphatic parts of the molecules.

In chapter 6 the lignans from sesame seeds were investigated. By making use of ESI-MS, as used for the detection of dibenzylbutane lignans (chapters 2-5), the lignans from sesame oil, sesamin and sesamolin could not be identified. Therefore, a new protocol for the analysis of lignans from sesame seeds in complex mixtures was developed. Analyzing several extracts from sesame seeds by APCI-MS and ESI-MS both in the positive and negative ionization mode resulted in such a protocol. It was concluded that a combination of APCI-MS in the positive and negative ionization mode was needed for the identification of all lignans in sesame seed extracts. The aglyconic lignans sesamin and sesamolin were identified in sesame oil, while the sesaminol glucosides were the main lignans found in the defatted sesame meal. The sesame lignans were subjected to a fermentation with human feces. Before they were fermented, they were enzymatically deglucosylated and acid treated. Fermentation resulted in the conversion of pinoresinol via SECO to END, but not in the conversion of sesamin, sesamolin and sesaminol. This supported the hypothesis that enterohepatic circulation is needed for the cleavage of the methylenedioxybridges.
In chapter 7, the results of this study are discussed. The opportunities of this study, especially with respect to the applicability of lignan macromolecule and monomeric lignans as health supplements, are discussed.

## Samenvatting

Lignanen zijn difenolische verbindingen die op basis van hun structurele overeenkomsten met het vrouwelijk geslachtshormoon estradiol en andere bekende estrogene verbindingen, functioneel geclassificeerd worden als fytoestrogenen. Lignanen worden in verband gebracht met een aantal gezondheidsbevorderende effecten als gevolg van hun interactie met het humane estrogeen metabolisme en hun antioxidant activiteit. De lignanen uit planten worden door bacteriën in de humane darm omgezet naar enterodiol (END) en enterolactone (ENL). Deze zogenaamde humane lignanen worden verantwoordelijk gehouden voor de effecten op de gezondheid omdat overwegend deze lignanen voorkomen in het lichaam (bloed en urine).
Het is bekend dat in vlaszaad lignanen gebonden zijn in een oligomere structuur, het lignaan-macromolecuul genoemd. Ondanks dat er onderzoek gedaan is naar welke verbindingen in het lignaan-macromolecuul voorkomen, is de structuur en samenstelling ervan grotendeels onbekend. Daarnaast is bekend dat lignanen door de bacteriën in de humane darm, omgezet worden in END en ENL, maar protocollen voor de productie van de humane lignanen middels fermentatie door een enkele bacterie soort zijn nauwelijks beschikbaar. Tevens ontbreken methoden voor het zuiveren van humane lignanen uit de bacterie-cultures. Om deze hiaten op te vullen, zijn voor dit onderzoek de volgende doelstellingen geformuleerd. De eerste doelstelling is om de exacte samenstelling en de structuur van het lignaan-macromolecuul uit vlaszaad te bepalen. Daarnaast wordt de omzetting van plantaardige naar humane lignanen door middel van fermentatie bestudeerd om de invloed van deze omzetting op de estrogeniciteit van de verbindingen te kunnen bepalen. Om deze onderzoeksvragen te kunnen beantwoorden, moeten nieuwe protocollen voor de analyse en zuivering ontwikkeld worden.

In hoofdstuk 1 wordt een algemene inleiding gegeven over de biosynthese, de bacteriële omzettingen en de bioactiviteit van lignanen. De lignanen, welke aanwezig zijn in vlas- en sesamzaad, worden beschreven. Daarnaast wordt een literatuuroverzicht over de invloed van lignanen op de gezondheid gegeven met speciale aandacht voor de invloed van lignanen op het estrogeen metabolisme en de antioxidant activiteit. Verder worden de (hypothetische) bacteriële omzettingsroutes van lignanen uit vlas- en sesamzaad naar de humane lignanen beschreven.
In hoofdstuk 2-4 worden de resultaten van het onderzoek naar de structuur van het lignaanmacromolecuul uit vlaszaad beschreven. Het kaf van vlaszaad werd gebruikt als bron omdat de concentratie lignanen in het kaf hoger is dan in geheel vlaszaad. Het lignaanmacromolecuul werd uit het kaf geëxtraheerd en afgebroken tot de monomere eenheden door middel van verzeping met 75 mM NaOH , zoals beschreven in hoofdstuk 2. Naast de al bekende componenten secoisolariciresinol diglucoside (SDG), p-coumaarzuur glucoside (CouAG) en ferulazuur glucoside (FeAG), werd het flavonoide herbactine diglucoside (HDG) met behulp van RP-HPLC-MS geïdentificeerd als een nieuw bestanddeel van het lignaan-macromolecuul. Nadat HDG op preparatieve schaal gezuiverd was, werd met behulp van NMR de identificatie bevestigd.

Om te bevestigen dat HDG daadwerkelijk deel uitmaakt van het lignaan-macromolecuul, werd het lignaan-macromolecuul gefragmenteerd. Door middel van partiële verzeping werden fragmenten verkregen die met behulp van RP-HPLC van elkaar gescheiden werden. Op basis van de MS data werd een fragment bestaande uit HDG en 3-hydroxy-3-methyl glutaarzuur (HMGA) geïdentificeerd. Met preparatieve RP-HPLC werd dit fragment gezuiverd en de identiteit met NMR bevestigd. Van HMGA is bekend dat het als verbindingsmolecuul functioneert tussen twee SDG moleculen. Op basis van het reeds genoemde fragment bestaande uit HDG en HMGA, kon worden geconcludeerd dat HDG op een vergelijkbare manier als SDG in het lignaan-macromolecuul gebonden is.
Partiële verzeping van het lignaan-macromolecuul bleek een essentiële behandeling om fragmenten te verkrijgen, die gebruikt kunnen worden bij de structuur opheldering van het lignaan-macromolecuul. Op basis van analytische RP-HPLC-MS werden sleutelfragmenten gevonden. Deze werden, na zuivering met preparatieve RP-HPLC, met NMR geïdentificeerd. Naast het fragment dat aantoonde dat HDG deel uitmaakt van het lignaanmacromolecuul, werden fragmenten gevonden waaruit bleek hoe CouAG en FeAG in het lignaan-macromolecuul gebonden zijn. Zoals in hoofdstuk 3 beschreven staat, zijn CouAG en FeAG via hun carboxyl-groepen aan het lignaan-macromolecuul gekoppeld. Een fragment werd geïdentificeerd waarin CouAG aan de C-6 van één van de glucose groepen van SDG gebonden zit. Echter, voor FeAG werd een dergelijk fragment niet gevonden. Wel werd een fragment gevonden bestaande uit ferulazuur (FeA) en SDG. In tegenstelling tot CouAG bleek FeA aan de C-2 positie van een glucose van SDG gebonden te zijn.
In hoofdstuk 4 worden de resultaten van het onderzoek naar de samenstelling en de grootte van het lignaan-macromolecuul beschreven. Op basis van de GC-gegevens werd bepaald dat het lignaan-macromolecuul voor $11 \%$ ( $\mathrm{w} / \mathrm{w}$ ) bestaat uit het verbindingsmolecuul HMGA. Van SDG, CouAG, FeAG en HDG werden de extinctie coëfficiënten bepaald. Deze zijn respectievelijk 5838, 10347, 19474 en $12133 \mathrm{M}^{-1} \mathrm{~cm}^{-1}$. Op basis van de piekoppervlaktes onder de RP-HPLC profielen en de extinctie coëfficiënten werd bepaald hoeveel SDG, CouAG, FeAG en HDG bijdragen aan de fractie fenolische componenten van het lignaan-macromolecuul. De fenolische fractie bestaat voor $56 \mathrm{~mol} \%$ uit SDG, en voor respectievelijk 23, 15 en $6 \mathrm{~mol} \%$ uit CouAG, FeAG en HDG.
Om de (gemiddelde) molecuulmassa te bepalen werd het lignaan-macromolecuul gefractioneerd met behulp van gelpermeatiechromatografie. Door het ontbreken van standaarden konden echter geen directe data over de molecuulmassa verkregen worden. Het feit dat het lignaan-macromolecuul als een brede piek elueerde, gaf wel aan dat het lignaanmacromolecuul gedefinieerd moeten worden als een verzameling moleculen van verschillende grootte. De samenstelling van de fracties werd bepaald door de verkregen fracties te verzepen en op RP-HPLC te analyseren. Het bleek dat het aandeel hydroxykaneelzuren (= CouAG + FeAG) negatief gecorreleerd is met de molecuulmassa. Dit leidde tot de hypothese dat als tijdens biosynthese een hydroxykaneelzuur aan het lignaan-macromolecuul gekoppeld wordt, de keten niet verder verlengd kan worden. Dit zou inhouden dat de hoeveelheid hydroxykaneelzuur wat tijdens de biosynthese aanwezig is, bepaalt hoe groot het lignaan-macromolecuul wordt.
Het MALDI-TOF MS spectra van het lignaan-macromolecuul liet een complex patroon van pieken zien. Hieruit bleek dat moleculen bestaande uit tenminste 5 SDG en 5 HMGA eenheden onderdeel uitmaken van het lignaan-macromolecuul.

De relatie tussen de ketenlengte van het lignaan-macromolecuul en de samenstelling daarvan werd beschreven in een model. De berekende gemiddelde ketenlengte is 3 SDG eenheden met aan beide uiteinden een hydroxykaneelzuur. De ketenlengte varieert tussen 1 en 7 SDG eenheden.

In hoofdstuk 5 wordt de omzetting van lignanen tijdens fermentatie bestudeerd. Secoisolariciresinol werd (SECO) verkregen door enzymatische deglucosilering en anhydrosecoisolariciresinol (AHS) door zuurbehandeling van volledig verzeept lignaanmacromolecuul. SECO en AHS verschillen in de structuur van hun alifatische groep. Het alifatische deel van SECO bestaat uit een diol-structuur terwijl in AHS deze diol-structuur is gedehydrateerd tot een furan-ring. SECO en AHS werden beide onderworpen aan fermentatie door bacteriën die in de dikke darm voorkomen of door aanverwante soorten. De methyl-groepen werden door Peptostreptococcus productus, Eubacterium limosum en Clostridium methoxybenzovorans van de methoxygroepen afgesplitst. In eerste instantie werd de enkelvoudig gedemethyleerde verbinding gevormd gevolgd door de tweevoudig gedemethyleerde vorm.
De tweevoudig gedemethyleerde producten werden in een grote batch fermentatie gevormd en gezuiverd met behulp van preparatieve RP-HPLC. Tweevoudig gedemethyleerd SECO werd vervolgens door Eggerthella lenta gedehydroxyleerd waardoor uiteindelijk END, één van de humane lignanen, gevormd werd. Gedurende de incubatie van tweevoudig gedemethyleerd AHS met Eg. lenta werden kleine hoeveelheden gedehydroxyleerde reactie-producten gevormd. De dehydroxylering van gedemethyleerd AHS verliep dus langzamer dan de dehydroxylering van gedemethyleerd SECO. De bacteriële omzettingsprocessen resulteerde in twee series verbindingen die verschillen van structuur in het alifatische deel van het molecuul.

In hoofdstuk 6 werden de lignanen uit sesamzaad bestudeerd. De ESI-MS procedure, die gebruikt werd voor de identificatie van dibenzylbutaan lignanen (hoofdstuk 2-5), bleek niet toereikend voor de identificatie van de lignanen sesamine en sesamoline uit sesamolie. Daarom moest een nieuw protocol ontwikkeld worden voor de analyse van lignanen uit sesamzaad. Verschillende extracten van lignanen uit sesamzaad werden geanalyseerd met ESI-MS en APCI-MS zowel in de positieve als in de negatieve ionisatie mode. Dit resulteerde in een nieuw protocol waarin APCI-MS in de positieve en in de negatieve mode gecombineerd moeten worden, om alle lignanen te kunnen identificeren die in een complex extract van sesamzaad voorkomen. Niet-geglucosileerde lignanen sesamine en sesamoline konden met deze methode in sesamolie geïdentificeerd worden. De sesaminol-glucosides werden geïdentificeerd als de meest voorkomende lignanen in ontvet sesammeel. Tevens werden ook andere lignanen, zoals pinoresinol, in sesamzaad aangetoond.
Lignanen uit sesamzaad werden geïncubeerd met humane feces. Voor fermentatie werden ze enzymatisch gedeglucosileerd en met zuur behandeld. Tijdens de fermentatie werd pinoresinol omgezet via SECO naar END. Echter, sesamine, sesamoline en sesaminol werden niet omgezet. Dit lijkt erop te duiden dat enterohepatische circulatie nodig is om de methyleendioxybruggen te verbreken.

In hoofdstuk 7 worden de behaalde resultaten bediscussieerd. De mogelijkheden die deze studie biedt, worden bediscussieerd met name met betrekking tot de toepasbaarheid van het lignaan-macromolecuul en de monomere lignanen als gezondheidsbevorderend supplement.

## Memories (Cats)

## Met trots presenteer ik u (Cabaret), mijn proefschrift getiteld <br> "Het lignaan macromolecuul uit vlaszaad"!

Zo zegeviert de wetenschap, in elk geval vandaag (Tarzan)! Ik heb het gedaan, het is me gelukt, stap voor stap, plan na plan (Cabaret). Ik wilde zien, ik wilde leren (Tarzan) en er was nog zoveel te doen en te weten, wat nog nooit was gezien of gedaan (Lion King). En dus lag een promotieonderzoek voor de hand, bij de leerstoelgroep Levensmiddelenchemie. Daar wilde ik mijn kunsten gaan vertonen (Evita). Daar had ik het naar mijn zin en voelde ik me thuis (Annie). Bij deze wil ik dan ook alle promovendi, analisten, studenten, docenten, professoren en Jolanda bedanken voor de beste werkomgeving die je je maar kan wensen.
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En laat het einde weer een nieuw begin zijn (Tarzan), want morgen behoort aan mij (Cabaret)!

Karin

## Curriculum Vitae

Karin Struijs was born on March 25, 1978 in Deventer (The Netherlands). After she graduated from secondary school (Scholengemeenschap "De Waerdenborch", Holten, The Netherlands), she came to Wageningen. In September 1996 she started her study Food Science at Wageningen University (The Netherlands).
From August 1999 till August 2000 she was a member of the board of student association SSR-W. She was in charge of one of the student dining halls of Wageningen. In September 2000 she continued her study Food Science with a major MSc-thesis at the Laboratory of Food Chemistry (Wageningen University), followed by a minor MSc-thesis in Toxicology (Wageningen University), an internship at the departments of Vegetable Crops and Pomology of UC Davis (CA, USA), and an industrial internship at Johma Nederland BV. She graduated in November 2002.
From December 2002 until November 2008 she worked on her PhD-research at the Laboratory of Food Chemistry of Wageningen University. The results of this research are described in this thesis. Beside the research, she was involved in several educational activities.

## List of publications

Van den Broek, L.A.M., Struijs, K., Verdoes, J.C., Beldman, G., Voragen, A.G.J., 2003. Cloning and characterization of two alpha-glucosidases from Bifidobacterium adolescentis DSM20083. Appl. Microbiol. Biotechnol. 61, 55-60.

Van der Woude, H., Gliszczynska-Swiglo, A., Struijs, K., Smeets, A., Alink, G.M., Rietjens, I.M.C.M., 2003. Biphasic modulation of cell proliferation by quercetin at concentration physiologically relevant in humans. Cancer Lett. 200, 41-47.

Kalamaki, M.S., Powell, A.L.T., Struijs, K., Labavitch, J.M., Reid, D.S., Bennett, A.B., 2003. Transgenic overexpression of expansin influences particle size distribution and improves viscosity of tomato juice and paste. J. Agric. Food Chem. 51, 7465-7471.

Struijs, K., Vincken, J-P., Verhoef, R., van Oostveen-van Casteren, W.H.M., Voragen, A.G.J., Gruppen, H., 2007. The flavonoid herbacetin diglucoside as a constituent of the lignan macromolecule from flaxseed hulls. Phytochemistry. 68, 1227-1235.

Struijs, K., Vincken, J-P., Verhoef, R., Voragen, A.G.J., Gruppen, H., 2008. Hydroxycinnamic acids are ester-linked directly to glucosyl moeities within the lignan macromolecule from flaxseed hulls. Phytochemistry. 69, 1250-1260.

Eeckhaut, E., Struijs, K., Possemiers, S., Vincken, J-P., De Keukeleire, D., Verstraete, W., 2008. Metabolism of the lignan macromolecule into enterolignans in the gastrointestinal tract, as determined in the simulator of the human intestinal microbial ecosystem. J. Agric. Food Chem., 56, 4806-4812.

Struijs, K., Vincken, J-P., Gruppen, H., Comparison of atmospheric pressure chemical ionization and electrospray ionization mass spectrometry for the detection of lignans from sesame seeds. Rapid Commun. Mass Spectrom. Accepted for publication.

Struijs, K., Vincken, J-P., Doeswijk, T., Voragen, A.G.J., Gruppen, H., The chain length of the lignan macromolecule from flaxseed hulls is determined by the incorporation of hydroxycinnamic acid glucosides. Submitted.

Struijs, K., Vincken, J-P., Gruppen, H., Bacterial conversion of anhydrosecoisolariciresinol. Submitted.

Mattinen, M.-L., Struijs, K., Suortti, T., Mattila, I., Kruus, K., Willför, S., Tamminen, T., Vincken, J.-P., Modification of lignans by Trametes hirsuta laccase. Submitted.

## Overview of completed training activities

## Discipline specific activities

## Conferences

Phytochemistry and Biology of Lignans, Walberberg, Germany, 2003
Symposium Lignans and Health, Helsinki, Finland, 2004
XXII International Conference on Polyphenols, Helsinki, Finland, 2004
European workshop Improving the Health Value of Plant Foods-Phytochemical Optimisation (COST926), Egmond aan Zee, The Netherlands, 2005
XXIII International Conference on Polyphenols, Winnipeg, Canada, 2006
Lignans, Alkylresorcinols and Health, Helsinki, Finland, 2007

## Courses

Ecophysiology of the Gastrointestinal tract, VLAG, Wageningen, The Netherlands, 2003
Chemistry and Biochemistry of Antioxidants, VLAG, Wageningen, The Netherlands, 2003
Bioinformation Technology 1, VLAG, Wageningen, The Netherlands, 2003
Protein engineering, VLAG, Wageningen, The Netherlands, 2004

## General courses

Supervision of Undergraduate Students, OWU, Wageningen, The Netherlands, 2003
PhD Introduction Week, VLAG, De Bilt, The Netherlands, 2003
Scientific writing, CENTA, Wageningen, The Netherlands, 2003
Conversation Skills, OWU, Wageningen, The Netherlands, 2005
"Food and Health", International Advanced Course, Socrates-VLAG, Paris, France, 2005

## Additional activities

Preparation PhD research proposal, Wageningen, The Netherlands, 2002
PhD Study trip, Japan, 2004
PhD Study trip, Belgium, France and England, 2006
Scientific Exchange Hamburg, Germany, 2004
Food Chemistry Seminars, Wageningen, The Netherlands, 2002-2008
Food Chemistry Colloquia, Wageningen, The Netherlands, 2002-2008


[^0]:    $\mathrm{ND}=\mathrm{n}$
    $[\mathrm{M}+\mathrm{Na}]$
    ${ }^{d}[\mathrm{M}+\mathrm{Na}] \quad[\mathrm{HAc}-\mathrm{H}]^{-}$; co-ionisation of analyte with acetate
    e - not possible to determine which $m / z$-ratio corresponded to the peak
    ${ }^{\mathrm{f}}$ low MS signals
    Since peak 12 and peaks $(14+15)$ behave differently (both in
    than $14+15$. Peak 12 is tentatively assigned as epi-sesaminone

[^1]:    ${ }^{\text {a }}$ incubation times of fermentation; t0 = start of fermentation, t24 = after 24 h of incubation, $\mathrm{t} 96=$ after 96 h of incubation
    ${ }^{\mathrm{b}} \mathrm{ND}=$ not detected in sample
    c - not possible to determine which $\mathrm{m} / \mathrm{z}$-ratio corresponded to the peak
    ${ }^{\mathrm{d}}$ Gray numbers indicate low MS signals
    ${ }^{e}$ negative ion mode does not correspond to positive ion mode. The positive ion mode was considered to be more reliable.
    ${ }^{\mathrm{f}}$ Assigned based on positive mode. $\mathrm{MS}^{2}$ fragmentation confirms this assignment.
    ${ }^{\mathrm{g}}$ Tentatively assigned as epi-sesaminone

